AWARD NUMBER: W81XWH-13-1-0441

TITLE: Transforming Research and Clinical Knowledge in Traumatic Brain Injury

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                                 Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution
                                 Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	26 Sep 2014 - 25 Sep 2015

4. TITLE AND SUBTITLE

Transforming Research and Clinical Knowledge in Traumatic Brain Injury

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0441

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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San Francisco, CA 94118-6215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Using the TRACK-TBI (*Transforming Research and Clinical Knowledge in TBI*) dataset we have created an Information Commons that integrates clinical, imaging, proteomic, genomic, and outcome biomarkers based upon the domains of the TBI Common Data Elements. The comprehensive TBI-CDE outcome measures allow for analyses of biomarker associations with a variety of measures. Available prognostic models have been evaluated against new prognostic models for TBI and found to be unsatisfactory using a multivariate approach that goes beyond the crude definitions of Mild, Moderate and Severe TBI. The latest neuroimaging methods including Quantitative CT, DTI, and resting-state functional MRI are surpassing other methods for predicting TBI patient outcomes. In emergency settings where high resolution neuroimaging is not available, rapid measurement of proteomic markers is appearing to be a valuable adjunct to current screening practices for ruling out TBI. Most importantly improving the collection of biomarkers in TBI patients will be vital to the design of future clinical trials.

15. SUBJECT TERMS
Traumatic Brain Injury; Common Data Elements; Prognosis; Outcomes

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			
Unclassified	Unclassified	Unclassified	Unclassified	92	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION**

   Traumatic Brain Injury (TBI) remains one of the greatest unmet needs in military and civilian medicine. The overall goal of the study is extensively analyze the *existing* data set from the multicenter pilot study entitled TRACK-TBI: *Transforming Research and Clinical Knowledge in TBI*. TRACK-TBI represents the largest multivariate TBI database across the injury spectrum from concussion to coma with CT/MRI imaging, blood biospecimens and outcome assessments. The DOD TRACK-TBI project is undertaking more extensive analysis of this highly granular cohort of TBI subjects. This work is vital to advancing our understanding of TBI and improving prognostic methods to identify individuals at risk for persistent cognitive and psychological heath disorders following TBI. This is being achieved by the following aims:

   Aim 1: To develop improved prognostic, diagnostic and outcome models for TBI.
   Aim 2: To identify neuroimaging biomarkers for diagnosis and prognosis in TBI.
   Aim 3: To identify proteomic and genomic associations with TBI phenotypes.

2. **KEYWORDS**

   Traumatic Brain Injury; Common Data Elements; Prognosis; Outcomes
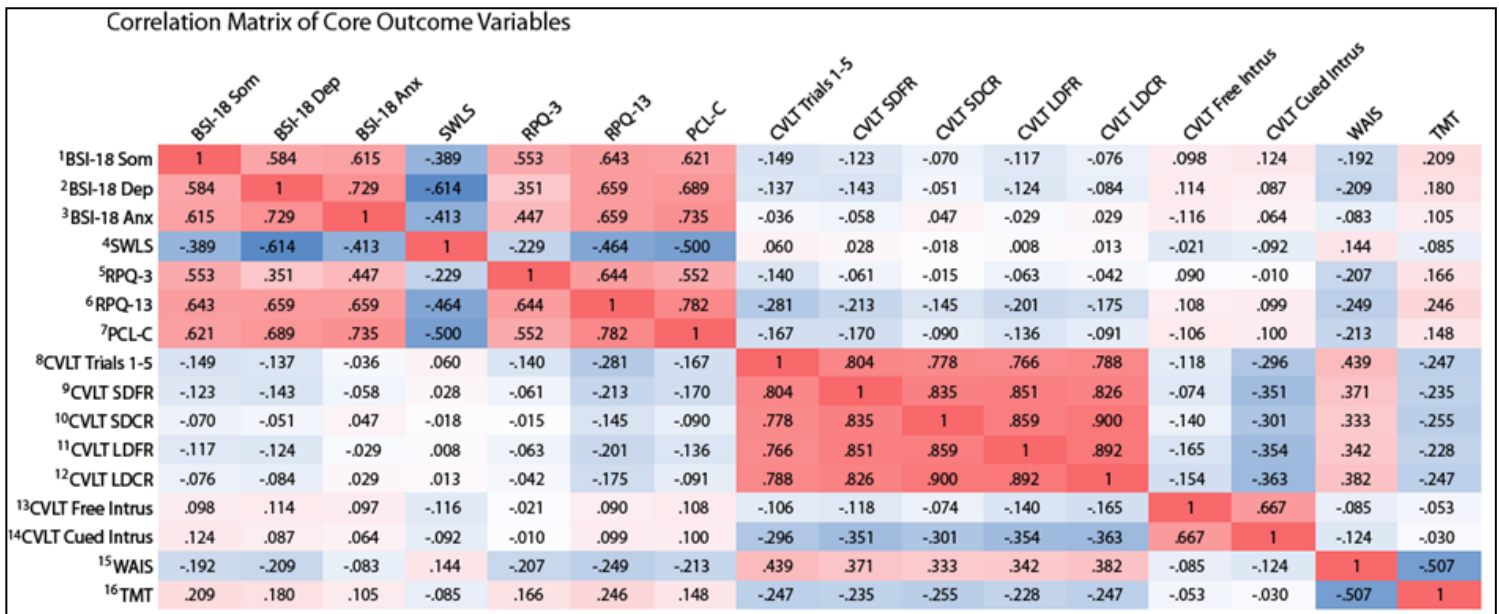
3. **OVERALL PROJECT SUMMARY**

   Progress Towards Aims 1-3:
   ***Aim 1: Diagnostic and Prognostic Outcome Models for TBI***

   Over the grant period to-date we have tested and cross-validated methods for dealing with missing values, testing outcome stability, and extracting multidimensional outcome patterns using the curated TRACK-TBI pilot dataset. A classic problem with TBI natural history studies and clinical trials is loss-to-follow-up, as well as variability in TBI phenotypes (Aim 1), neuroimaging (Aim 2), genomics and proteomics (Aim 3) that can skew data, leading to prognostic models that lack stability and cross-validity. Indeed this has been shown by Lingsma et al. (2015), funded by the current DoD project. However, modern bootstrapping and permutation approaches have potential to help by simulating much larger populations. Permutation methods use random sampling with replacement at the level of univariate (i.e., single variables) and multi-dimensional outcomes (i.e., multiple variables and their co-variances). Machine learning analytics provide a powerful methodological toolkit for performing permutation analysis. For example, modern Topological Data Analysis (TDA) and non-linear principal components analysis (NL-PCA) provide a means to test for outcome stability across the full set of endpoints in TRACK-TBI pilot, in the face of loss-to-follow-up and heterogeneity in endpoint availability. We have applied these methods to demonstrate the generalizability of emerging predictive models based on the TRACK-TBI pilot. Permutation approaches to outcome prediction have yielded the following reportable findings on outcomes in the past quarter:

   • Across the full outcome set in TRACK-TBI pilot there is substantial cross-correlation among outcome instruments that can be discerned by NL-PCA **(Figure 1).**
   • At a multidimensional level, the outcome correlation matrix partitions into separable domains consisting of a psychosocial outcome set (PC1), global verbal memory set (PC2), recall errors (PC3), and processing speed (PC4) **(Figure 2)**.
   • These data-driven outcome domains are highly stable and robust against missing values and are stable across 2000 iterations of population subsampling (bootstrapped permutation test; see "progress detail" below).
   • The distinct outcomes domains are differentially sensitive to the impact of TBI as measured by CT.

- The verbal memory outcome set is more sensitive to CT-detected TBI severity than the Glasgow Outcome Scale Extended (GOS-E)(Cohen's d=0.52 for verbal memory vs. d=0.34 for GOS-E). **(Figure 3).**
- Together, the computational work indicates that multidimensional outcomes are more sensitive to early predictors such as CT pathology than traditional GOS-E, suggesting that high-resolution combinatorial endpoint monitoring can contribute to improved prognostic and diagnostic outcome modeling.

Correlation Matrix of Core Outcome Variables

	BSI-18 Som	BSI-18 Dep	BSI-18 Anx	SWLS	RPQ-3	RPQ-13	PCL-C	CVLT Trials 1-5	CVLT SDFR	CVLT SDCR	CVLT LDFR	CVLT LDCR	CVLT Free Intrus	CVLT Cued Intrus	WAIS	TMT
[1] BSI-18 Som	1	.584	.615	-.389	.553	.643	.621	-.149	-.123	-.070	-.117	-.076	.098	.124	-.192	.209
[2] BSI-18 Dep	.584	1	.729	-.614	.351	.659	.689	-.137	-.143	-.051	-.124	-.084	.114	.087	-.209	.180
[3] BSI-18 Anx	.615	.729	1	-.413	.447	.659	.735	-.036	-.058	.047	-.029	.029	-.116	.064	-.083	.105
[4] SWLS	-.389	-.614	-.413	1	-.229	-.464	-.500	.060	.028	-.018	.008	.013	-.021	-.092	.144	-.085
[5] RPQ-3	.553	.351	.447	-.229	1	.644	.552	-.140	-.061	-.015	-.063	-.042	.090	-.010	-.207	.166
[6] RPQ-13	.643	.659	.659	-.464	.644	1	.782	-.281	-.213	-.145	-.201	-.175	.108	.099	-.249	.246
[7] PCL-C	.621	.689	.735	-.500	.552	.782	1	-.167	-.170	-.090	-.136	-.091	-.106	.100	-.213	.148
[8] CVLT Trials 1-5	-.149	-.137	-.036	.060	-.140	-.281	-.167	1	.804	.778	.766	.788	-.118	-.296	.439	-.247
[9] CVLT SDFR	-.123	-.143	-.058	.028	-.061	-.213	-.170	.804	1	.835	.851	.826	-.074	-.351	.371	-.235
[10] CVLT SDCR	-.070	-.051	.047	-.018	-.015	-.145	-.090	.778	.835	1	.859	.900	-.140	-.301	.333	-.255
[11] CVLT LDFR	-.117	-.124	-.029	.008	-.063	-.201	-.136	.766	.851	.859	1	.892	-.165	-.354	.342	-.228
[12] CVLT LDCR	-.076	-.084	.029	.013	-.042	-.175	-.091	.788	.826	.900	.892	1	-.154	-.363	.382	-.247
[13] CVLT Free Intrus	.098	.114	.097	-.116	-.021	.090	.108	-.106	-.118	-.074	-.140	-.165	1	.667	-.085	-.053
[14] CVLT Cued Intrus	.124	.087	.064	-.092	-.010	.099	.100	-.296	-.351	-.301	-.354	-.363	.667	1	-.124	-.030
[15] WAIS	-.192	-.209	-.083	.144	-.207	-.249	-.213	.439	.371	.333	.342	.382	-.085	-.124	1	-.507
[16] TMT	.209	.180	.105	-.085	.166	.246	.148	-.247	-.235	-.255	-.228	-.247	-.053	-.030	-.507	1

Figure 1. Outcome cross-correlations represented as a heat map where positive correlations are red and negative correlations are blue. Note that outcomes instruments cluster together into modules.
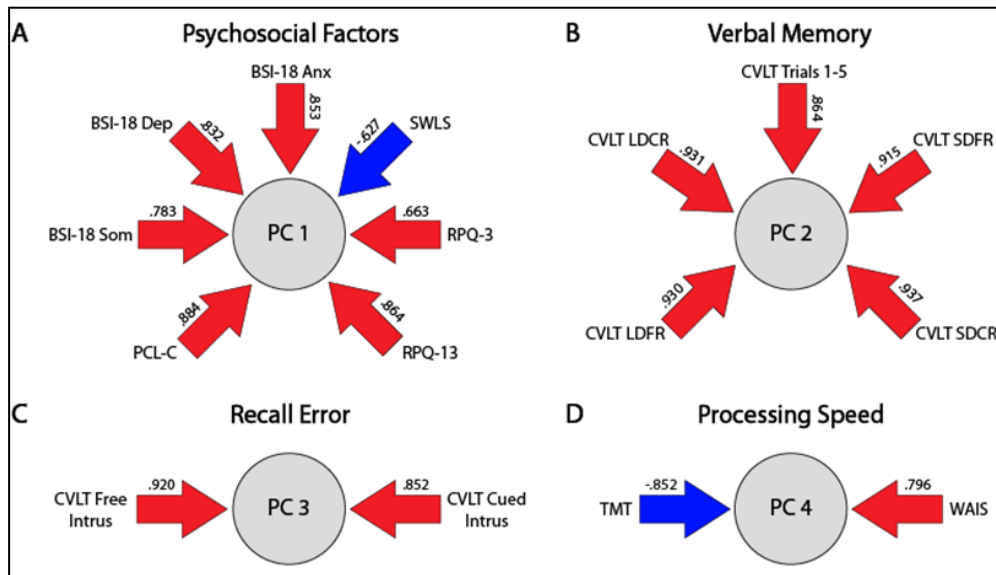
Figure 2. Principal component analysis partitions PCs into separable clusters of outcome metrics that move together as emergent patterns. PCs are represented as circles and arrows reflect PC loadings (red positive relationship, blue inverted relationship). Based on loading patterns the PCs represent: A) Psychosocial Factors, B) Verbal Memory, C) Recall Errors, and D) Processing Speed. These PC outcome modules may be differentially sensitive to the impact of TBI.
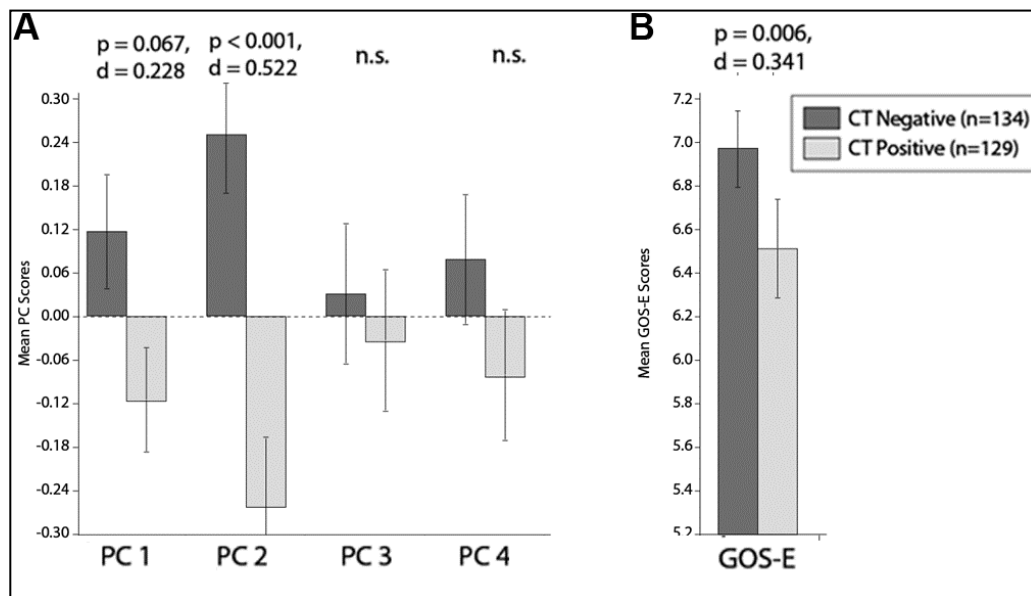
Figure 3. To test the sensitivity of PCs to TBI, we calculated patient scores on PC1-4 and their sensitivity to positive head CT findings using a general linear model and Cohen's d for effect size (a measure of sensitivity). A) PC2 (verbal learning) was significantly sensitive to positive head CT and demonstrated a high effect size (d=.52). B) GOS-E also demonstrated sensitivity to positive head CT, however it demonstrated lower effects size than PC2 (d=.34). The results suggest that multidimensional verbal memory is more sensitive than GOS-E as an outcome measure to TBI.

Aim 2: *Neuroimaging biomarkers for diagnosis and prognosis in TBI*

We have applied multidimensional approaches to the analysis and integration of neuroimaging biomarkers across both CT and MRI domains. In addition we have worked to develop novel TDA approaches for diffusion tensor imaging. The following reportable findings come from our analysis of imaging biomarkers and their integration with phenotypes (Aim 1) and molecular biomarkers (Aim 3):

CT Pathology Domains. Over the past year we have developed and cross-validated a novel application of non-linear (categorical) principal component analysis (NLPCA).
    • We used NLPCA to integrate 20 CT endpoints from 207 TRACK-TBI patients for hypothesis testing of biomarkers that predict the severity of each cluster of pathology.
        • We found that 5 PCs that accounted for more than 70% of the variance in the dataset.
        • We now have a preliminary set of distinct CT pathology domains from these patients that can be used for hypothesis testing on blood biomarkers that predict these features, ranging from general brain pathology following TBI to very specific types of pathology such as diffuse axonal injury.

MRI Topological Data Analysis (TDA) Map and Expression of ANKK1, COMT, APOE E4
    • Using the multimodal Allen Human Brain Atlas which integrates anatomic and genomic information and MRI data for we have a full transcriptome associated with each MRI voxel in X,Y,Z. In addition we have annotations reflecting the anatomical region of each transcriptome and anatomical mapping to the Montreal Neurological Institute (MNI) brain atlas space.
    • We then projected the expression levels of ANKK1 and COMT into the MRI TDA map to determine the anatomical location of ANKK1 and COMT expression in the human brain.
    • It was found that two genes are expressed in different MRI coordinate locations. This suggests that these distinct molecular pathways have distinct locations in the human brain. Combined with our observation that the ANKK1 T/T and COMT G/G show up in two different subpopulations of mild TBI, (both of which have higher rates of PTSD and lower GOSE) the results suggest that we would want to simultaneously look at both SNPs in a multplexed biomarker array to have coverage of the TBI syndromic space to predict outcome.

Aim 3. *Proteomic and genomic associations with TBI phenotypes.* Over the past year we have harnessed multidimensional analytics and ensemble machine learning approaches to biomarker panels analyzed as part of the current DoD grant. In particular we applied ensemble machine learning approaches to multi-analyte assays of TRACK-TBI pilot samples. This contributed to the recently published paper on ANKK1 (Yue et al., 2015) which demonstrated that the genetic biomarker ANKK1 predicts 6 month verbal outcome after TBI as well as related papers (Sorani et al,. 2015) and submitted work (Winkler et al., submitted). The following list summarizes reportable outcomes:

    • ANKK1 predicts 6 month verbal outcome after TBI on the California Verbal Learning Test.
    • Through the use permutation tests, ANKK1 effect on TBI outcome can be disentangled from natural ethic and racial variation in ANKK1 that would typically confound outcome prediction.
    • This suggests that ANKK1 may be a high-resolution genetic biomarker of outcomes when used in combination with ensemble analytic approaches.

Subtask 4: Development of a preliminary prognostic model for mild TBI
Progress: The prognostic modeling is moving ahead with a novel approach based upon the multimodal Allen Human Brain Atlas which integrates anatomic, genomic and MRI data resulting in a full transcriptome associated with each MRI voxel in X,Y,Z. In addition we have annotations reflecting the anatomical region of each transcriptome and anatomical mapping to the Montreal Neurological Institute (MNI) brain atlas space.

We harnessed the data to address the questions:
  • Where are ANKK1 and COMT expressed in the human brain?
  • Are these anatomically redundant biomarkers or orthogonal markers that should be used in conjunction to predict dopaminergic tone/syndromic outcome in TBI?

We then projected the expression levels of ANKK1 and COMT into the MRI TDA map to determine the anatomical location of ANKK1 and COMT expression in the human brain. To construct **Figure 4**, we extracted the topological map of the X,Y,Z MRI coordinate space co-registered to the MNI brain atlas space. The nodes represent locations and the edges represent adjacent MRI locations (in X,Y,Z simultaneously). We then projected the expression levels of ANKK1, COMT and APOE-E4 alleles into the MRI TDA map to determine the anatomical location of expression of these transcripts in the human brain. Note that the 3 genes are expressed in different MRI coordinate locations. This suggests that these distinct molecular pathways have distinct locations in the human brain. Combined with our observation that the ANKK1 T/T and COMT G/G show up in two different subpopulations of mild TBI, (both of which have higher rates of PTSD and lower GOSE) the results suggest that we would want to simultaneously look at both SNPs in a multiplexed biomarker array to have coverage of the TBI syndromic space to predict outcome.

Figure 4. Projected expression levels of ANKK1 and COMT and APO-E onto the Allen Brain Atlas MRI co-registered to the MNI coordinates. TDA was used to map the anatomical location of ANKK1 and COMT expression in the human brain.
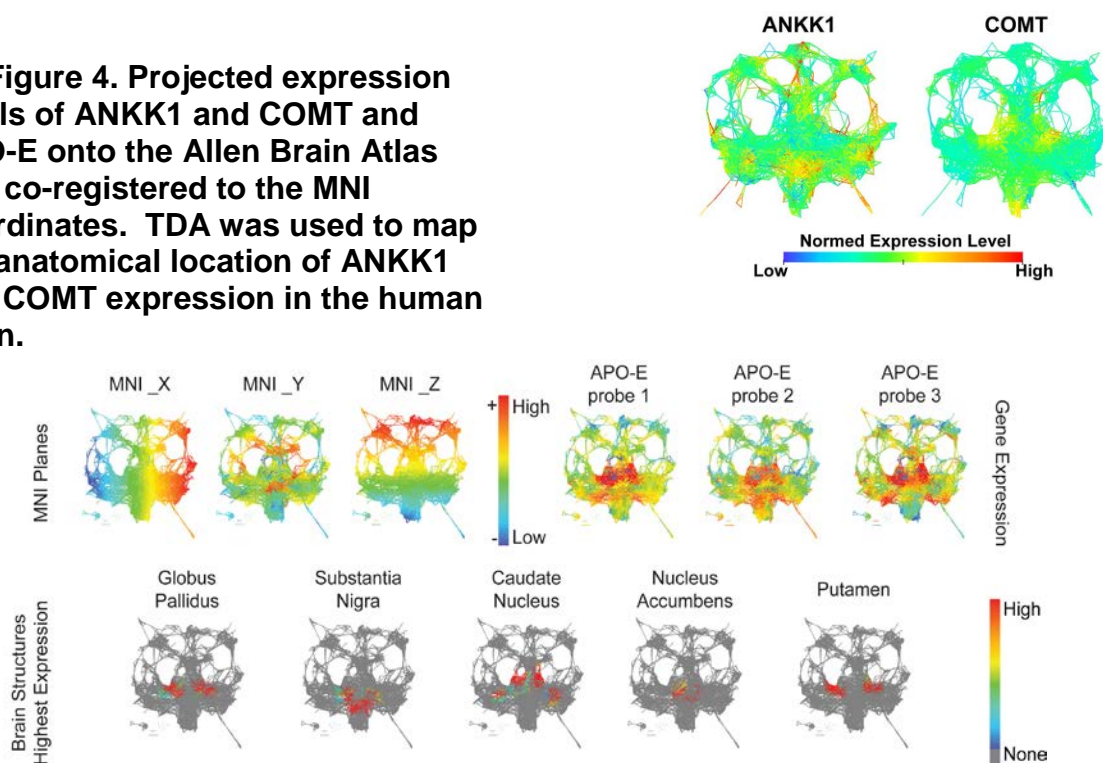
"Progress Detail"

Aim 1: Diagnostic and Prognostic Outcome Models for TBI

We have applied a large scale missing values analysis and multiple imputation approaches to discover consistent multidimensional outcome patterns in neurocognitive and psychiatric outcome patterns in the face of patient drop-out and missing values (Cooper et al., in preparation). A fundamental issue with outcome prediction is that a large percentage of TBI population may be lost to follow-up. There is an open question about whether this drop-out is random or systematic, and its bias in clinical trials populations remains unclear. The modern statistical science of Missing Values Analysis (MVA) provides a toolkit to test the impact of missing-ness on outcome patterns in TBI. To test this we carefully examined missing values patterns in the TBI pilot dataset **(Figure 5)**. As can be clearly seen, there are missing values that accumulate across the patient population resulting in a winnowing of the 'complete' dataset from N=599 down to N=263 with complete data across all variables at 6 months. However, N=381 patients with some outcomes and N=545 with some variables, but no useable 6 month outcomes. Fortunately modern multiple imputation methods allow us to precisely measure the degree of uncertainty that accumulates across this typical drop-out pattern. <u>This work has identified consistent features of neurocognitive outcome assessment that are stable in the face of random variation in patient follow-up and outcome across the full spectrum of TBI severity.</u>
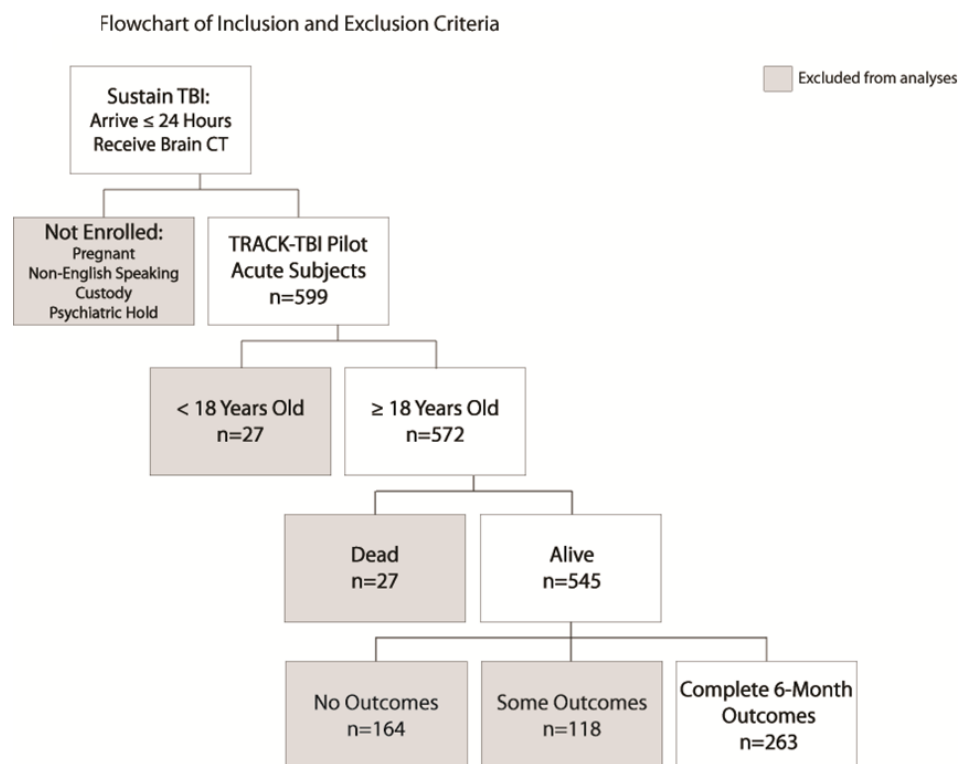
Figure 5. Determining sources of 'missing data' in TRACK-TBI Pilot. Clinical TBI populations have several sources of missing data that accumulate over the duration of any given study. Missing Values Analysis (MVA) and Multiple Imputation (MI) methods can help assess whether these missing data are random or systematic, and can help determine the bias and cross-validity of findings.

To directly test the effect of patient drop-out on outcome patterns we performed a combined analysis of all of the alive patients (N=545). Initial missing values analysis suggested that there were significant baseline differences in the patient sub-population that completed the 6-month outcomes (N=263) relative to the patients with missing outcomes **(Figure 6A)**. Specifically, patients with missing values were significantly older and had a marginally-significant difference in CT scans. However, no differences were observed in GCS and GOS-E between the patient subpopulations with missing values and complete values. Altogether, this suggests a modest bias due to patient dropout. To explicitly test whether missingess skewed multidimensional outcome patterns we used modern expectation maximization methods to generate 10 different imputed datasets. When we reanalyzed these data and confirmed that multiple imputation eliminated any bias in the dataset at the univariate level **(Figure 6B)**. This suggested that multiple imputation may be used to help correct for bias in TBI populations to achieve a more representative patient sample.

A

Variable	Groups	Test	Sig.
Age	M/I (n=282) Comp (n=263)		.012*
GCS	M/I (n=280) Comp (n=263)	Mann-Whitney U Test	.874
GOS-E	M/I (n=118) Comp (n=263)		.160
CT Pathology	M/I (n=282) Comp (n=263)	Chi-Squared	.050*

B

Variable	Variable Type	F	Sig.
Age	Baseline	.396	.949
CT Pathology		.190	.997
GCS		.004	1.000
GOS-E	GOS-E	.440	.927
BSI-18 Som	Psychosocial Factor (PC 1)	.066	1.000
BSI-18 Dep		.141	.999
BSI-18 Anx		.135	.999
SWLS		.089	1.000
RPQ-3		.163	.998
RPQ-13		.101	1.000
PCL-C		.222	.994
CVLT Trials 1-5	Verbal Memory (PC 2)	.285	.985
CVLT SDFR		.351	.967
CVLT SDCR		1.107	.352
CVLT LDFR		.726	.701
CVLT LDCR		.620	.798
CVLT Free Intrus	Recall Error (PC 3)	1.072	.380
CVLT Cued Intrus		.463	.914
WAIS	Processing Speed (PC 4)	.721	.705
TMT		.185	.997

Figure 6. Assessing the impact of 'missing data' in TRACK-TBI Pilot. A) We performed a careful missing values analysis to test whether there were differences between the TBI population with missing/incomplete (M/I) data versus the complete data (Comp). Significant differences were observed in Age and CT pathology, suggesting a potential bias if we only analyze 'complete' data, but not with respect to GCS and GOS-E, suggesting that these measures are 'blind' to bias in the TBI population. B) We next applied 10 iterations of multiple imputation according to modern statistical science regarding missing data and expectation-maximization (EM) algorithms (Rubin and Little, 2002), and reanalyzed the pooled, imputed data. After imputing for missing data, there was no observed bias across the populations with M/I versus Complete data at either the univariate or multivariate level.

To further test whether the TRACK-TBI pilot is a representative TBI population with respect to multidimensional outcomes we stratified patients into Mild and Moderate/Severe injuries by GCS and then re-extracted PC patterns within each of these populations. To ensure that we

detected stable patterns given limited N's from the subpopulations, we performed a bootstrapping exercise where we compared NL-PC patterns, sampled with replacement 2000 times within each injury substratification, each time drawing 75% of the subpopulation. The results suggest that mild and moderate severe populations have essentially the same multidimensional patterns, however PC1 and PC2 flip positions in the moderate to severe injuries with verbal accounting for a higher proportion of the variance explained that psychosocial factors (whereas psychosocial factors account for more variance in the mild TBI).

Bootstrapped Rotated Component Matrix of Mild TBI				
Outcome Variable	Psychosocial Factors (PC 1)	Verbal Memory (PC 2)	Recall Error (PC 3)	Processing Speed (PC 4)
BSI-18 Som	.586	.160	.060	.073
BSI-18 Dep	.612	.149	.069	.004
BSI-18 Anx	.660	.235	.029	.004
SWLS	-.501	-.162	-.139	.042
RPQ-3	.491	.145	-.079	.106
RPQ-13	.609	.088	.018	.066
PCL-C	.632	.114	.036	.029
CVLT Trials 1-5	.136	.621	-.065	-.056
CVLT SDFR	.158	.666	-.087	-.037
CVLT SDCR	.220	.705	-.012	-.026
CVLT LDFR	.174	.680	-.092	-.023
CVLT LDCR	.209	.696	-.113	-.030
CVLT Free Intrus	.065	.030	.865	.064
CVLT Cued Intrus	-.026	-.206	.800	.049
WAIS	-.048	.154	-.112	-.200
TMT	.048	-.104	-.024	-.217

Illustration of each variable's principal component score for principal components 1-4 using the bootstrapped data from the mild TBI (GCS 13-15) cohort. Data was bootstrapped 2000 times using 75% of the dataset's total cases, and then aggregated by taking the mean.

Figure 7. Comparison of PC loading patterns in Mild (top) and Moderate/Severe (bottom) TBI. Note that the loading patterns are essentially the same. However the rank ordering of PC1 and PC2 flip between the two injury severities. Because PCs are rank ordered in descending variance explained, this flip indicates that psychosocial factors account for more variance in mild TBI whereas verbal memory accounts for more variance in moderate to severe TBI.

Bootstrapped Rotated Component Matrix of Moderate and Severe TBI				
Outcome Variable	Verbal Memory (PC 2)	Psychosocial Factors (PC 1)	Recall Error (PC 3)	Processing Speed (PC 4)
BSI-18 Som	-.034	.685	.154	.151
BSI-18 Dep	.004	.816	.056	-.022
BSI-18 Anx	-.034	.812	.018	-.013
SWLS	-.192	-.356	.034	.071
RPQ-3	-.065	.574	-.055	.092
RPQ-13	-.163	.805	-.018	.002
PCL-C	.147	.839	.103	.052
CVLT Trials 1-5	.852	-.171	-.037	.009
CVLT SDFR	.855	-.041	-.069	-.031
CVLT SDCR	.884	.150	-.090	-.011
CVLT LDFR	.928	.000	-.071	-.032
CVLT LDCR	.924	.069	-.080	-.021
CVLT Free Intrus	-.385	.087	.475	.171
CVLT Cued Intrus	-.496	-.025	.417	.128
WAIS	.551	-.121	.035	.120
TMT	-.108	.000	-.306	-.268

Illustration of each variable's principal component score for principal components 1-4 using the bootstrapped data from the moderate/severe TBI (GCS ≤12) cohort. Data was bootstrapped 390 times (n=39, bootstrapped by a factor of 10) using 75% of the dataset's total cases, and then aggregated by taking the mean. Though Psychosocial Factors and Verbal Memory switch between principal component 1 and 2 as compared to the other cohorts, loading patterns of outcome measures were conserved.

Non-Bootstrapped Rotated Component Matrix of Entire Cohort

Outcome Variable	Psychosocial Factors (PC 1)	Verbal Memory (PC 2)	Recall Error (PC 3)	Processing Speed (PC 4)
BSI-18 Som	.783	-.057	.045	-.116
BSI-18 Dep	.832	-.050	.087	-.062
BSI-18 Anx	.853	.034	.037	.057
SWLS	-.627	-.052	-.160	.047
RPQ-3	.663	-.037	-.150	-.152
RPQ-13	.864	-.167	-.010	-.125
PCL-C	.884	-.093	.033	-.031
CVLT Trials 1-5	-.110	.864	-.057	.183
CVLT SDFR	-.082	.915	-.093	.103
CVLT SDCR	.013	.937	-.038	.108
CVLT LDFR	-.058	.930	-.105	.082
CVLT LDCR	-.008	.931	-.130	.134
CVLT Free Intrus	.074	-.033	.920	-.014
CVLT Cued Intrus	.054	-.300	.852	.033
WAIS	-.148	.292	-.090	.796
TMT	.131	-.147	-.092	-.852

Illustration of each variable's principal component score for principal components 1-4 using the non-bootstrapped data from the entire sample.

Figure 8. PC Loading patterns in the entire cohort is more consistent with of Mild TBI.

Altogether the results suggest that multidimensional outcome patterns are largely stable in TRACK-TBI pilot and are likely to generalize to larger populations. These results largely support Aim 1, and indicate that we are making strong headway toward better prognostic and diagnostic outcome models through our multidimensional analytic approaches.

Aim 2: Neuroimaging biomarkers for diagnosis and prognosis in TBI

We used a Region of Interest (ROI) method to analyze diffusion tensor imaging (DTI) data from mild TBI (TBI) patients from the TRACK-TBI (Transforming Research and Clinical Knowledge in Traumatic Brain Injury) pilot study. Although prior studies have reported white matter injury, as DTI metrics, within the acute-to-subacute time period after mild traumatic brain injury (mTBI), there has been an unresolved and striking inconsistency across studies in the *direction* of reported alterations in the DTI metrics. _We show that changes in DTI metrics, as a manifestation of microstructural white matter injury, demonstrate geographic, tract-specific variations throughout the brain._

- We found not only that the *magnitude* of changes in DTI metrics demonstrate tract-specific variations throughout the brain after mTBI, but also that *both increases* and *decreases* in FA can occur in the subacute time frame following mTBI.
- The only statistically significant *increases* in fractional anisotropy (FA) in mTBI patients were observed in *anterior tracts* (anterior corona radiata, $p = 3 \times 10^{-6}$; anterior limb of internal capsule, $p = 8 \times 10^{-4}$; superior fronto-occipital fasciculus, $p = 0.03$) while the only significant *decreases* in FA were observed in *posterior tracts* (splenium of corpus callosum, $p = 7 \times 10^{-8}$; posterior corona radiata, $p = 0.03$; posterior limb of internal capsule; $p = 0.06$).
- To demonstrate that *"large-throughput image analysis in combination with the use of data-driven analytical methods, can aid in uncovering relationships among imaging pathoanatomic features and prognosis,"* we also used topological data analysis techniques to demonstrate the presence of striking subdivisions or substructures within traditional tractography-defined tracts. We showed that effect sizes of the altered FA in mild TBI patients

vary across the different substructures. This suggests a **basis for new ROI schemes for DTI analysis**, *based on a data-driven and more detailed parcellation of white matter than is currently available from widely used white matter atlases.*

<u>*DTI and fMRI region-of-interest (ROI) analysis.*</u>   In addition to whole-brain voxelwise nonparametric permutation testing, we will perform complementary hypothesis-driven ROI analyses of the TRACK-TBI DTI and resting-state fMRI data. ROIs that have been frequently implicated in white matter injury in TBI will be delineated using the Johns Hopkins University (JHU) ICBM-DTI-81 White Matter Labeled Atlas. We will perform nonparametric statistical analyses to compare patient and control groups for each ROI. The use of ROI analysis addresses the possibility that a whole-brain voxelwise approach might be less sensitive in mild TBI patients, in whom the extent and severity of white matter injury are more limited."

> *"Hypothesis1b: Objective computational tools can be used to analyze conventional neuroimaging studies in acute TBI and, through large-throughput image analysis in combination with the use of data-driven analytical methods, can aid in uncovering relationships among imaging pathoanatomic features and prognosis"*

Region-of-interest (ROI) analysis of fractional anisotropy (FA)
   Figures 9 and 10 show comparisons of FA among mTBI subjects from the TRACK pilot study and control subjects, based on the JHU ICBM-DTI-81 white matter atlas. <u>*We demonstrate there are opposing directions of the alterations in FA in mTBI patients relative to control subjects, based on location within the brain.*</u> Specifically, several anterior tracts demonstrated statistically significant <u>*decreases*</u> in mTBI compared to control subjects. Among these regions with <u>*decreased*</u> FA in mTBI versus control subjects, the largest size effect was seen within the anterior corona radiata. Statistically significant reductions were also noted in the anterior limbs of the internal capsules and superior fronto-occipital fasciculi.   In contrast, statistically significant <u>*increases*</u> were found to occur only within posterior white matter tracts. In particular, there was a highly significant <u>*increase*</u> in FA within the splenium in mTBI compared to control subjects.
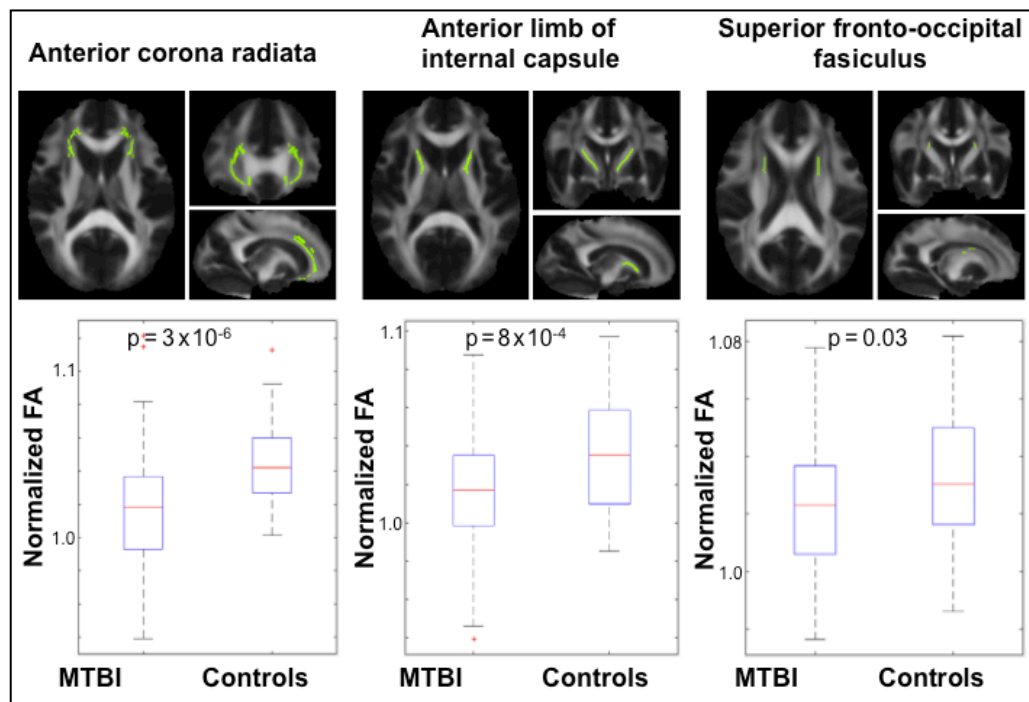
Figure 9. **Anterior white matter tracts demonstrate lower normalized FA in mTBI patients relative to control subjects.** Of all white matter tracts and regions investigated, only the anterior corona radiata (ACR), anterior limb of internal capsule (ALIC), and superior fronto-occipital fasciculus (SFO) demonstrated FA values that were statistically significantly *decreased* in mTBI patients relative to control subjects at $p < 0.05$. (*On each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, and outliers are plotted individually.*)
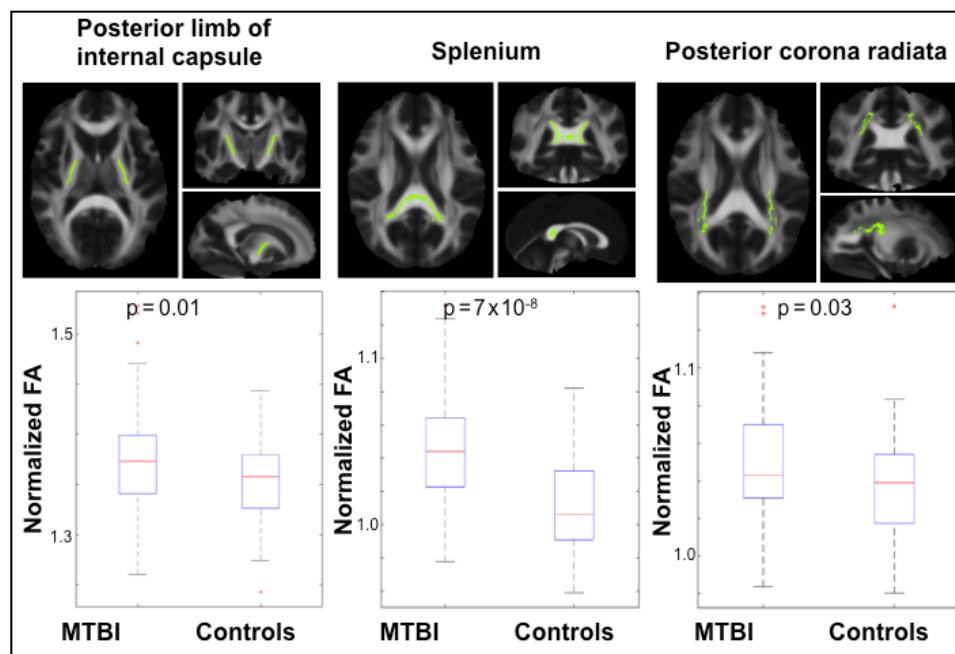
Figure 10. **Posterior white matter tracts demonstrate lower normalized FA in mTBI patients relative to control subjects.** Of all white matter tracts and regions investigated, only the posterior limb of internal capsule (ALIC), splenium (SCC), and posterior corona radiata (PCR) demonstrated FA values that were statistically significantly *increased* in mTBI patients relative to control subjects at $p < 0.05$. (*On each box, central mark is the median, edges of the box are 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, and outliers are plotted individually.*)

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Substructures within JHU ROIs

For several of the JHU regions, we used topological data analysis to reveal striking internal color patterns for combinations of parameters as shown in **Figures 11 and 12**. Specifically, these figures show JHU regions colored by the principal components 1 and 2 (PC1 and PC2) of the fractional anisotropy. They demonstrate unexpected internal substructures within several brain regions. The presence of these correlations on a voxelwise level suggests subdivision of the JHU regions into substructures or subdivisions based on internal microstructural, and possibly functional, organization.
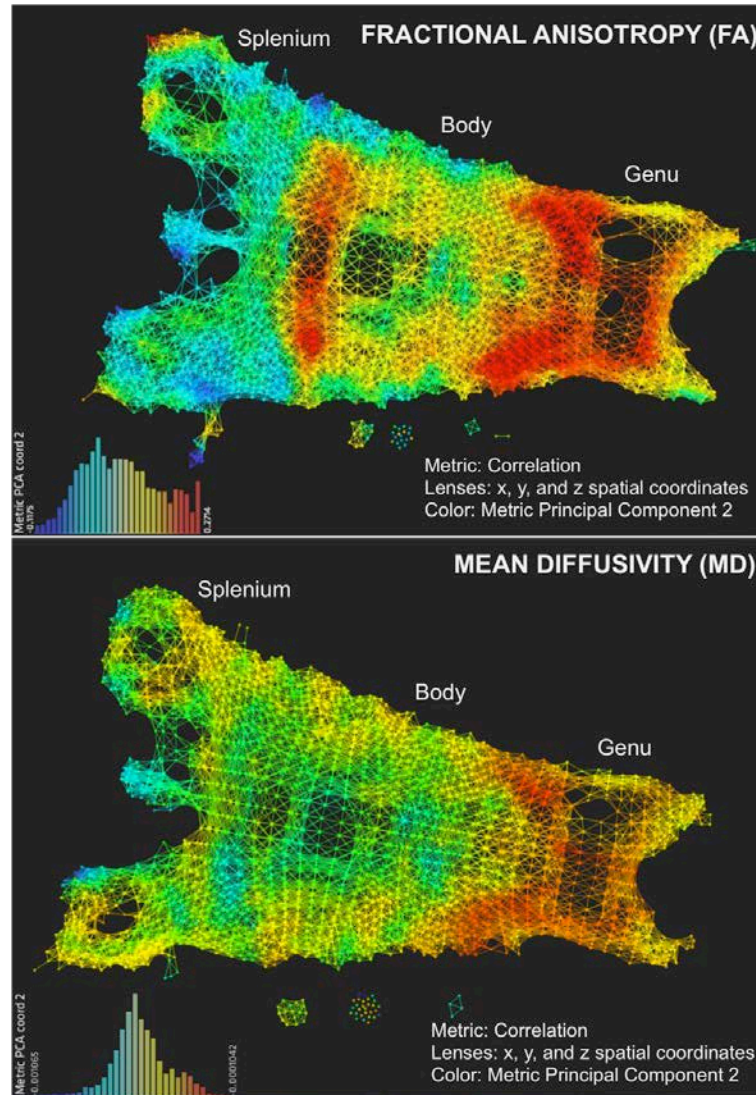
Figure 11. Networks of white matter voxels *within* the corpus callosum. We identifiied striking internal subdivisions within the corpus callosum. **(Top)** DTI fractional anisotropy (FA) values within regions with similar colors are highly correlated with one another across human subjects. The presence of these correlations on a voxelwise level is consistent with internal microstructural and functional substructures within the corpus callosum. The regions identified through this topological data analysis are much more detailed than the traditional division of the callosum into genu, body and splenium. **(Bottom)** DTI mean diffusivity (MD) values also demonstrate striking internal patterns, though different from those for the FA.

A Principal Component 1 (mean FA):
All mTBI and controls

B Principal Component 2
All mTBI and controls

C Effect size of FA difference:
Complicated mTBI versus controls
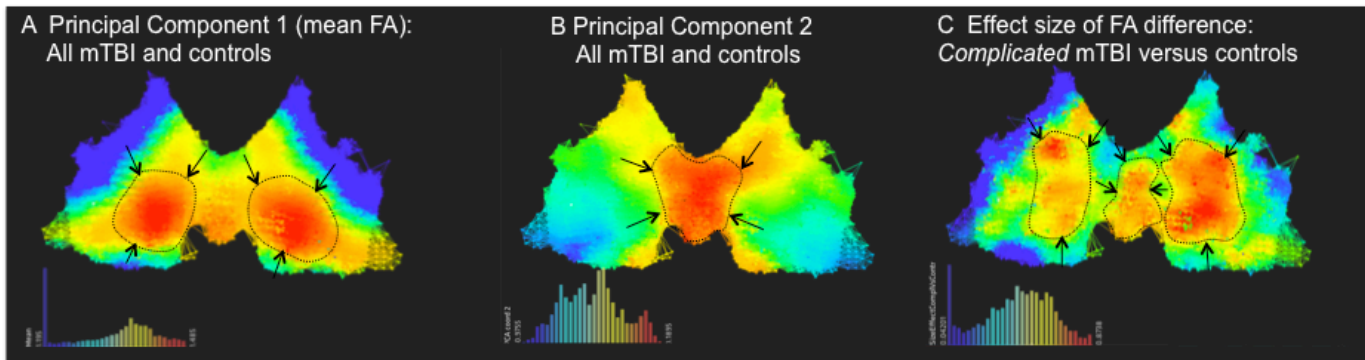
Figure 12 in particular shows that there also exist regular geographic patterns in the *size effect* of FA differences between mTBI and control subjects. **Figure 12** depicts the splenium of the corpus callosum. **Figures 12A and 4B** show the spatial dependence of the magnitude of Principal Components 1 and 2 within the splenium. Comparison of **Figures 12A and 12B** to **Figures 12C** demonstrates that the largest effect sizes in FA group differences are located within the *same regions with the highest magnitudes of PC1 and PC2*. The observation that subdivisions within the splenium that consist of highly coherent FA values, based on their similar PC1 and PC2 values (and manifested as regions of *uniform color* on **Figures 12A and 12B**), also demonstrate the highest effect sizes in the alterations of those FA values after injury suggests a promising parcellation scheme for ROIs that may be more sensitive for traumatic white matter microstructural changes.

Aim 3: Proteomic biomarker panels as a potential diagnostic and prediction tool

We performed multi-analyte proteomic biomarker analysis on 61 proteins in 131 TRACK-TBI subjects. We found three principal components (PC1-3) to test the diagnostic and predictive validity of these proteomic modules by testing for statistical associations with imaging biomarkers (intracranial CT), clinical diagnostic tools (GCS motor score) and neurocognitive outcomes (CVLT at 6 months). PC1 reflected an inverted relationship between inflammatory cytokine markers and apolipoproteins. PC2 reflected a proteome biomarker signature reflecting the covariance between antigen presenting cell markers (CD40) inflammatory markers (TNF; IL-23), coagulation factors and apolipoproteins. PC3 reflects the covariance between neurotrophins (BDNF), markers of neuronal damage (NSE) and inflammatory markers involving the T-cell and B-cell acquired immune system (CD-40L, RANTES) as well as the innate immune system (ENA). To further test the potential clinical utility of the principal proteomic components, we derived each patient's individual score within the proteome space defined by PC1-3. Using these unique scores, we then tested for the statistical predictive association between the proteome patterns and admission CT findings, 6 month CVLT and Admission GCS. The rationale for testing these 3 sets of clinical variables is that they reflect the distinct domains imaging biomarkers, clinical tools, and neurocognitive outcomes. PC1 and PC3 (but not PC2) were significantly associated with positive head CT findings. PC1 and P2 (but not PC3) were significantly predictive of CVLT standard scores (trial 1-5) at 6 months. PC1 (but not PC2 or PC3) were significantly predictive of Admission GCS motor score (dichotomized to < 4). Altogether the data suggest that PC1, in particular is broadly predictive of TBI imaging, the current

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'gold standard' clinical TBI diagnostic tool, and long term neurocognitive outcome. These preliminary results provide strong preliminary support for proteomic biomarker panels as a potential diagnostic and prediction tool. Further work is required to cross validate these results and to assess their ROC characteristics.
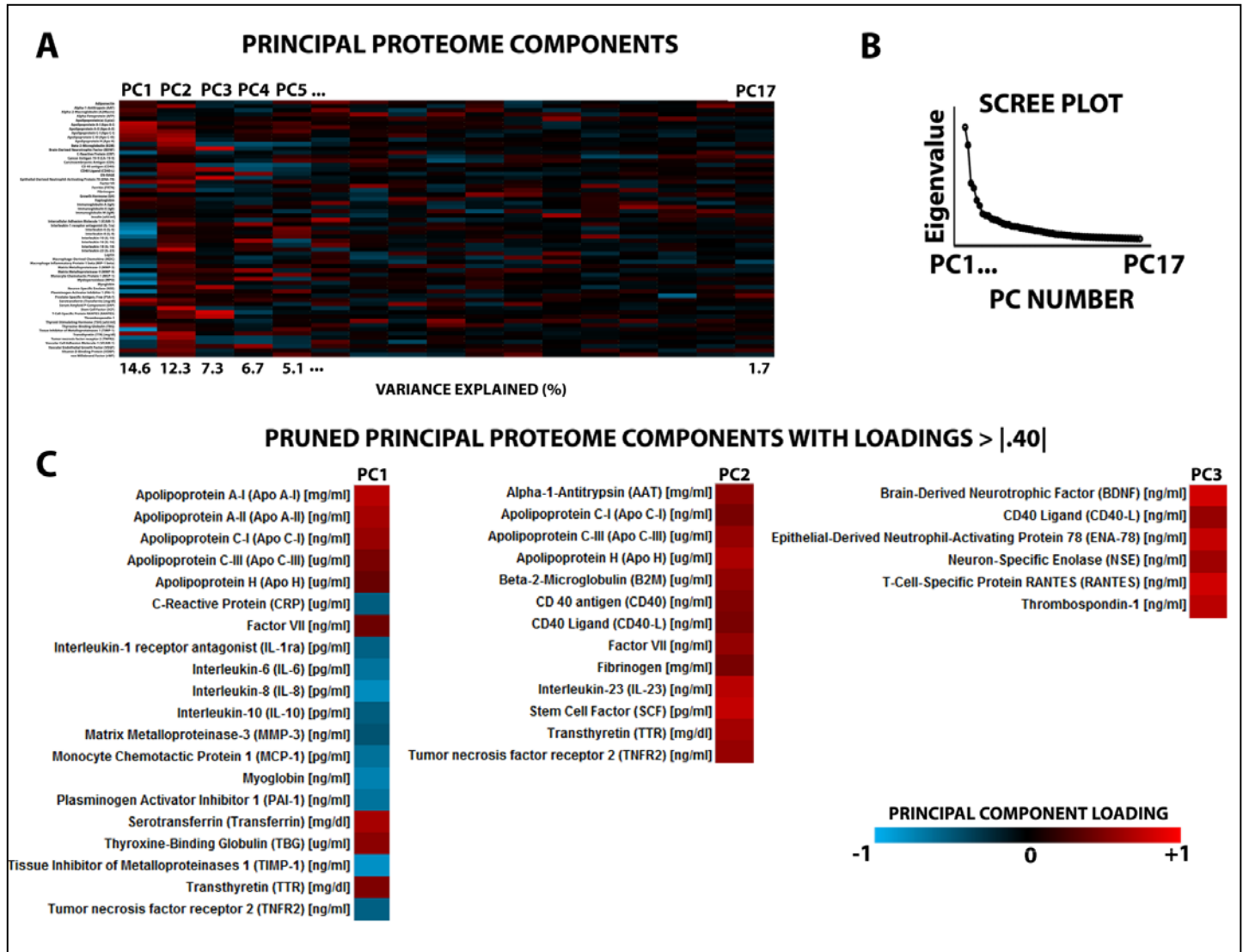

Figure 13. NL-PC Loading patterns from proteomic biomarker panels. A) NL-PCA on a massively multiplexed proteome array, extracted 17 principal components that met the eigenvalue>1 retention rule. B) A Screeplot allowed us to pruned this NL-PCA solution down to 3 PCs for full interpretation. C) Examination of loadings greater than |.45| indicated that PC1 reflects a broad cluster of inflammatory Proteomic biomarkers.

Predictor Variable	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
CT FINDINGS	PC1	28.591	1	28.591	36.255	.000
	PC2	.156	1	.156	.154	.696
	PC3	5.119	1	5.119	5.259	.023
6 month CVLT	PC1	64.015	43	1.489	3.418	.000
	PC2	43.922	43	1.021	2.120	.006
	PC3	49.420	43	1.149	1.334	.163
Admission GCS Motor	PC1	32.468	1	32.468	42.816	.000
	PC2	.036	1	.036	.036	.850
	PC3	1.336	1	1.336	1.332	.251

Figure 14. Association of NL-PC proteome patterns from acute biomarker samples with CT findings (diagnostic), 6 month CVLT (prognostic) and admission GCS motor score (diagnostic). Note that PC1 is significantly associated with all 3 of these important endpoints, suggesting its potential as an powerful diagnostic and prognostic biomarker set.

4. **KEY RESEARCH ACCOMPLISHMENTS**
   - Together our computational models indicate that multidimensional outcomes are more sensitive to early predictors such as CT pathology than traditional GOS-E, suggesting that high-resolution combinatorial endpoint monitoring can contribute to improved prognostic and diagnostic outcome modeling.
   - For subjects with complicated mild TBI with 3T MRIs performed at 2 weeks post injury we used region of interest (ROI) and whole-brain voxelwise approaches to identify extensive areas of white matter injury in complicated mild traumatic brain injury (MTBI) patients. Although prior studies have reported white matter injury, as evidenced by alterations in diffusion tensor imaging (DTI) metrics, within the acute-to-subacute time period after mTBI, there has been an unresolved and striking inconsistency across studies in the *direction* of reported alterations in the DTI metrics. _We show that changes in DTI metrics, as a manifestation of microstructural white matter injury, demonstrate geographic, tract-specific variations throughout the brain._
   - Over the past year we have harnessed multidimensional analytics and ensemble machine learning approaches to multi-analyte assays of TRACK-TBI pilot samples. This contributed to the recently published paper on ANKK1 (Yue et al., 2015) which demonstrated that the genetic biomarker ANKK1 predicts 6 month verbal outcome after TBI as well as related finding in recently published paper on COMT (Winkler et al.,2015). These findings suggest that ANKK1 may be a high-resolution genetic biomarker of outcomes when used in combination with ensemble analytic approaches.
   - The prognostic modeling is moving ahead with a novel approach based upon the multimodal Allen Human Brain Atlas which integrates anatomic, genomic and MRI data resulting in a full transcriptome associated with each MRI voxel. In addition we have annotations reflecting the anatomical region of each transcriptome and anatomical mapping to the Montreal Neurological Institute (MNI) brain atlas space. We projected the expression levels of ANKK1, COMT and APOE-E4 alleles into the MRI Topographical Data Analysis map to determine the anatomical locations of expression in the human

brain. The 3 genes are expressed in different MRI coordinate locations suggesting that these distinct molecular pathways have distinct locations in the human brain. Combined with our observation that the ANKK1 T/T and COMT G/G show up in two different subpopulations of mild TBI, (both of which have higher rates of PTSD and lower GOSE) the results suggest that we would want to simultaneously look at both SNPs in a multiplexed biomarker array to have coverage of the TBI syndromic space to predict outcome.

5. CONCLUSIONS

We have demonstrated progress across all aims which are of significance to civilian and military TBI clinical care. We have successfully created an Information Commons for the TBI CDE domains across the injury spectrum from concussion to coma. We have developed more sensitive image analysis tools and imaging biomarkers to better diagnose and predict mTBI patients that will have an unfavorable outcome. We have also identified new genetic markers in the dopamine pathway that appear to contribute to unfavorable outcome after TBI. This opens the possibility of pre-deployment and pre-injury (sports and occupational) screening for individuals at risk for unfavorable outcome following TBI. We have also clearly established the multifactorial nature of TBI and the need for a combination of clinical, imaging and blood-based marker for the diagnosis and prediction of outcome. Most importantly these markers will be vital to the design of future clinical trials and treatment for TBI.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

Published During Year 2 (Provided in Appendix):

Sorani MD, Yue JK, Sharma S, Manley GT, Ferguson AR; TRACK-TBI Investigators. Genetic Data Sharing and Privacy. Neuroinformatics. 2015 Jan;13(1):1-6. PMID 25326433.

Korley FK, Diaz-Arrastia R, Wu AH, Yue JK, Manley GT, Sair HI, Van Eyk J, Everett AD; TRACK-TBI Investigators. Circulating Brain-Derived Neurotrophic Factor Has Diagnostic and Prognostic Value in Traumatic Brain Injury. J Neurotrauma. 2015 Sep 18. Epub ahead of print. PMID 26159679.

Yue JK*, Pronger AM*, Ferguson AR, Temkin NR, Sharma S, Rosand J, Sorani MD, McAllister TW, Barber J, Winkler EA, Burchard EG, Hu D, Lingsma HF, Cooper SR, Puccio AM, Okonkwo DO, Diaz-Arrastia R, Manley GT; COBRIT Investigators; TRACK-TBI Investigators. Association of a Common Genetic Variant within ANKK1 with Six-Month Cognitive Performance After Traumatic Brain Injury. Neurogenetics. 2015 Jul;16(3):169-80. PMID 25633559.

Wang KK, Yang Z, Yue JK, Zhang Z, Winkler EA, Puccio AM, Diaz-Arrastia R, Lingsma HF, Yuh EL, Mukherjee P, Valadka AB, Gordon WA, Okonkwo DO, Manley GT; TRACK-TBI Investigators. Plasma Anti-Glial Fibrillary Acidic Protein (GFAP) Autoantibody Levels During the Acute and Chronic Phases of Traumatic Brain Injury – A TRACK-TBI Pilot Study. *J Neurotrauma* 2015 Nov 11. [Epub ahead of print]

Winkler EA*, Yue JK*, McAllister TW, Temkin NR, Oh SS, Burchard EG, Hu D, Ferguson AR, Lingsma HF, Burke JF, Sorani MD, Rosand J, Yuh EL, Barber J, Tarapore PE, Gardner RC, Sharma S, Satris GG, Eng C, Puccio AM, Wang KK, Mukherjee P, Valadka AB, Okonkwo DO, Diaz-Arrastia R, Manley GT; TRACK-TBI Investigators. COMT Val158Met Polymorphism is

Associated with Nonverbal Cognition Following Mild Traumatic Brain Injury. *Neurogenetics.* 2015 Nov 17. [Epub ahead of print]

Manuscripts In Press or Under Review:
Pirracchio R, Yue JK, Manley GT, van der Laan MJ, Hubbard AE; TRACK-TBI Investigators. Collaborative Targeted Maximum Likelihood Estimation (cTMLE) for Variable Importance Measure: Illustration for Functional Outcome Prediction in Mild Traumatic Brain Injuries. In Press, Stat Methods Med Res. Accepted 2015 Sep 13.

Haarbauer-Krupa J, Taylor C, Yue JK, Winkler EA, Cooper SR, Stein MB, Manley GT; TRACK-TBI Investigators. Screening for Post-Traumatic Stress Disorder in a Civilian Emergency Department Population with Traumatic Brain Injury. Submitted to J Neurotrauma

Abstracts and Presentations:

AMERICAN CONGRESS OF REHABILITATION MEDICINE, OCT 2015, DALLAS, TX
Haarbauer-Krupa J, Taylor C, Yue JK, Winkler EA, Cooper SR, Stein MB, Manley GT; TRACK-TBI Investigators. Screening for Post-Traumatic Stress Disorder in a Civilian Emergency Department Population with TBI. (Oral Presentation)

CONGRESS OF NEUROLOGICAL SURGEONS, SEP 2015, NEW ORLEANS, LA
Yue JK, Winkler EA, McAllister TW, Temkin NR, Ferguson AR, Lingsma HF, Sorani MD, Rosand J, Wang KK, Gardner RC, Yuh EL, Barber J, Tarapore PE, Sharma S, Burchard EG, Hu D, Eng C, Mukherjee P, Valadka AB, Okonkwo DO, Diaz-Arrastia R, Manley GT; TRACK-TBI Investigators. COMT Val158Met Polymorphism is Associated with Domain-Specific Cognitive Impairment Following Mild TBI. (Oral Presentation)

Winkler EA, Yue JK, Ferguson AR, Temkin NR, Stein MB, Barber J, Yuh EL, Sharma S, Satris GG, McAllister TW, Rosand J, Sorani MD, Lingsma HF, Vassar MJ, Tarapore PE, Burchard EG, Hu D, Eng C, Puccio AM, Wang KK, Mukherjee P, Valadka AB, Okonkwo DO, Diaz-Arrastia R, Manley GT; TRACK-TBI Investigators. COMT Val158Met Polymorphism is Associated with Post-Traumatic Stress Disorder and Outcome Following Mild TBI. (Forum Presentation)

NATIONAL NEUROTRAUMA SYMPOSIUM POSTERS, JUL 2015, SANTA FE, NM

Yue JK, Robinson CK, Winkler EA, Ferguson AR, McAllister TW, Rosand J, Lingsma HF, Sharma S, Sorani MD, Cooper SR, Nielson JL, Satris GG, Vassar MJ, Korley FK, Wang KK, Yuh EL, Mukherjee P, Valadka AB, Okonkwo DO, Diaz-Arrastia RR, Manley GT; TRACK-TBI Investigators. APOE E4 is Associated with Decreased Six-Month Verbal Memory Performance After Mild TBI.

Yue JK, Sharma S, Winkler EA, Vassar MJ, Rick JW, Ratcliff JJ, Adeoye OM, Ferguson AR, Lingsma HF, Korley FK, Satris GG, Robinson CK, Yuh EL, Mukherjee P, McAllister TW, Diaz-Arrastia RR, Valadka AB, Gordon WA, Okonkwo DO, Manley GT; TRACK-TBI Investigators. Temporal Profile of Care Following Mild Traumatic Brain Injury: Predictors to Hospital Admission, Outpatient Referral and Outcome.

Sharma S, Yue JK, Winkler EA, Robinson CK, Ratcliff JJ, Adeoye OM, Ferguson AR, Rick JW, Korley FK, Vassar MJ, Yuh EL, Mukherjee P, McAllister TW, Diaz-Arrastia RR, Valadka AB, Gordon WA, Okonkwo DO, Manley GT; TRACK-TBI Investigators. Outpatient Referral at 3-Months is Associated with 6-Month Symptomatology Following Mild Traumatic Brain Injury.

Winker EA, Yue JK, Ferguson AR, McAllister TW, Rosand J, Tarapore PE, Vassar MJ, Wang KK, Mukherjee P, Valadka AB, Okonkwo DO, Diaz-Arrastia RR, Manley GT; TRACK-TBI Investigators. DRD2 C957T Polymorphism is Associated with Improved 6-Month Verbal Learning Following Traumatic Brain Injury.

7. INVENTIONS PATENTS AND LICENSES
None

8. REPORTABLE OUTCOMES
None.

9. OTHER ACHIEVEMENTS
None.

10. REFERENCES
None

11. APPENDICES
Published Manuscripts during Year 2

Genetic Data Sharing and Privacy

**Marco D. Sorani · John K. Yue · Sourabh Sharma · Geoffrey T. Manley · Adam R. Ferguson ·
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Keywords Data · Privacy · Genetic · Biospecimen ·
Biobank · Sharing

Capitalizing on the Promise of Genetic Data

Genetic data has provided valuable insights into disease
cause and risk as well as drug discovery and develop-
ment in neuroscience. For example, human genetics
studies have provided insights into cognition (Glahn
et al. 2013) and psychiatric disorders (Kao et al.
2010). The genetic basis of several inherited disorders
such as Down's Syndrome and Tay-Sachs disease are
well known, and other associations such as the role of
APOE in Alzheimer's disease are still extensively stud-
ied. However, despite advances in understanding the
human genome, there are concerns about the privacy
of genetic data and potential discrimination resulting
from its disclosure, and there has been incomplete over-
sight of genetic testing (Scheuner et al. 2008).

At the same time, there have been increased efforts
to share research data to enable scientific discovery and
achieve cost efficiencies. It has become clear that no
scientist can guarantee absolute privacy, and it is also
increasingly recognized that research will work better if
scientists have more information about the people they

study and that being identifiable has some benefits
(Angrist 2013). There are examples of pioneering efforts
in neuroscience research. The fMRI Data Center is a
leader in open-access data sharing in the functional
neuroimaging community, overcoming logistical, cultural
and funding barriers (Mennes et al. 2013). Similarly, the
INCF Task Force on Neuroimaging Datasharing has
started work on tools to ease and automate sharing of
raw, processed, and derived neuroimaging data and
metadata (Poline et al. 2012).

In the United States, legislation such as the Health
Insurance Portability and Accountability Act (HIPAA)
(Gostin 2001) and the Genetic Information
Nondiscrimination Act have attempted to limit access
to sensitive data and discrimination related to health
insurance and employment, but it has been known for
over a decade that seemingly anonymized data can be
related to publicly available information to identify spe-
cific individuals (Braun et al. 2009) using diagnosis
codes (Tamersoy et al. 2010), rare visible disorders
(Eguale et al. 2005), allele frequencies (Craig et al.
2011), place and date of birth (Acquisti and Gross
2009), a combination of a surname with age and state
(Gymrek et al. 2013), and patient health location visit
patterns (Malin 2007). Re-identification methods have
included genotype-phenotype inferences, family struc-
tures, and dictionary attacks (Malin 2005).

In total, these facts have changed the goals of many
research organizations from making data re-identification
impossible to making it highly improbable and educat-
ing stakeholders about the issues and risks, while en-
hancing research collaborations by sharing data. Here,
we discuss data privacy and sharing approaches, we
provide recommendations and describe our own experi-
ences in the context of biobanking, and we look ahead

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to address challenges and opportunities for data privacy and sharing.

Data Sharing "Carrots" and "Sticks"

Data sharing is often driven by a set of incentives and consequences. Benefits include a desire to "democratize" data, an evolution towards more "big science" collaboration, a desire to minimize the burden on research participants, technical developments such as web based databases, standardized data sharing guidelines, and opportunities for exclusivity in manuscript submission and citation of data sets. For example, the "data paper" allows researchers to publish their datasets as a citable scientific publication, gives credit that is recognizable within the scientific community, and ensures the quality of the published data and metadata through the peer review process (Gorgolewski et al. 2013). Consequences and risks of not sharing data include declining financial resources, a need to manage data beyond the lifecycle of a grant, and requirements by journal editors. Challenges to effective sharing remain, including the removal of disincentives for data sharing by industry, the reduction of litigation risks, the availability of patient level data, and the willingness to foster discussion in cases of differences of interpretation of data.

Privacy Strategies, Processes and Technologies

To address privacy objectives, a wide variety of strategies have been proposed to protect sensitive data. Many organizations rely on the Safe Harbor Standard of the HIPAA Privacy Rule, which enumerates 18 identifiers that must be suppressed (Malin et al. 2011), though HIPAA has also been criticized by the Institute of Medicine for provisions that seem to hinder data access while failing to provide substantive privacy protection (Franc et al. 2011). De-identification procedures have been applied to a variety of types of free-text data, including electronic health records (Meystre et al. 2010), laboratory and clinical narrative reports (Friedlin and McDonald 2008), discharge and order summaries (Aberdeen et al. 2010), and pathology reports (Thomas et al. 2002), as well as non-text data formats such as images (Gonzalez et al. 2010) and geocode data (Cassa et al. 2006). Johnson et al. tested a method for generating global unique identifiers to link data and specimens by sending encrypted information to a server application with over 8000 individuals in an autism study. They implement a balance between distinguishing individuals to gain research insights and protecting confidentiality (Johnson et al. 2010).

A recent survey of biopharma companies found that the most common data coding practice was de-identification. Only 10 % of companies anonymized their data. Most reported retaining a secondary key internally either by a party independent of the one responsible for de-identification or by the same party but with restricted access (38 %). De-identification programs are difficult to implement, cumbersome, costly, inefficient and offer little added privacy protection (Franc et al. 2011).

Privacy processes and technologies have evolved together. Processes include establishing an "honest broker" that provides investigators with de-identified or limited datasets under stipulations contained in a signed data use agreement (Liu et al. 2009), and establishing data enclaves where investigators apply to obtain restricted access to data for a limited time with the understanding they will be monitored (Rodgers and Nolte 2006). Some of these strategies may be difficult to implement and explain to patients and other stakeholders, and they may not provide added benefit proportional to the cost. At the same time, many automated algorithms for anonymizing data are now available (Loukides et al. 2010). Algorithms include family relation linking (Malin 2006), encryption (Landi and Rao 2003), and hiding functions (Huang et al. 2010). In addition, database software systems are a mature, increasingly ubiquitous technology and come with robust security and audit functionality. For example, a recent public epidemiology project implemented a database system for data privacy using a multi-layered role and right-of-access control plus de-identification (Meyer et al. 2012). Researchers, particularly in academia, must be prepared to move past spreadsheets.

Biobanks as Laboratories for Data Sharing

The emergence of genomic technologies has spurred rapid growth in the collection of biosamples and the development of biobanks. While some types of research data can be useful even if they are "permanently" anonymized, biosample data poses unique challenges for security from a research perspective. Genetic data must be kept private as stipulated in informed consent agreements, but other related data does not face this requirement or must in fact remain identifiable. To achieve patient confidentiality, DNA samples can be re-labeled with unique identifiers that are different than the identifiers initially assigned in a clinical trial. This re-labeling or double-coding process is referred to as de-identification. With data de-identification, the data belonging to an individual in the clinical environment can still be associated with the same individual in a de-identified research context. An increasingly common research objective involves building analytical databases using de-identified clinical data while enabling the data set to be updated with new pseudonymous data over time (Noumeir et al. 2007).

In the United States, biobanks in academia, government, and industry have implemented a range of sharing and

security practices. At Vanderbilt University, a DNA biobank linked to data from an electronic medical record (EMR) system implemented a de-identified mirror image of the EMR, a policy of extracting DNA from discarded blood samples, revision of standard consent, and procedures for de-identification. About 700 to 900 samples were added per week (Roden et al. 2008). The National Mesothelioma Virtual Bank is based on the caTISSUE Clinical Annotation Engine developed in cooperation with the Cancer Biomedical Informatics Grid and includes paraffin embedded tissues, tissue microarrays, serum and genomic DNA. It provides real-time access to de-identified data depending on user authorization (Amin et al. 2008). Internationally, practices also vary. The Genome Austria Tissue Bank developed data protection tools and considered ethical, legal and social issues as it changed from a population-based tissue bank to a disease-focused biobank (Asslaber et al. 2007). The National Cancer Center Hospital in Tokyo established a bio-repository in 2002 for both de-identified and non-de-identified post-clinical test samples. A portion of samples are transferred to new tubes before and after being frozen. This transfer is the only de-identification procedure (Furuta et al. 2011).

Recommendations and Examples

For those new to multi-center studies and 'big data' analyses of genomic and clinical data, several guiding principles may be useful. Other resources such as the NIH's "Data Sharing Policy and Implementation Guidance" and the NSF's "Data Sharing Policy" should also be consulted.

- Institutional review board (IRB) approval of study designs, informed patient consent of study participation, and understanding of HIPAA requirements are essential.
- A flexible and powerful computing platform for data management is critical but does not have to be complex or expensive. Emailing spreadsheets will lead to problems – setting up an open source database with web access has become increasingly feasible.
- There are generally four categories of coding for data security: identified, coded (including single-coded and double-coded or "de-identified"), anonymized, and anonymous. It is necessary to select the appropriate level of data coding to balance patient security and research purposes.
- Providing data alone is not enough to enable effective sharing – data dictionaries describing the data and, of course, human support are critical. Common analytical methods and tools further support sharing of metadata and results.
- Realistic expectations regarding sharing (e.g., database updates and speed, publication plans, etc.) and privacy

(e.g., who will have access to what information, etc.) will establish trust.
- Thinking ahead to future requirements (e.g., will investigators want to obtain consent to re-contact subjects for follow-up?) and challenges (e.g., is there a plan in case of data security breach?) will enhance likelihood of overall success and sustainability.

In general, collaboration with a cross-functional team of clinicians, statisticians, geneticists, informaticians, and other relevant subject matter experts will also help identify opportunities and challenges.

An Example of Multi-Dimensional Data Sharing: TRACK-TBI

Data-sharing for precision-medicine in neuroscience will require co-mingling of biorepository, brain imaging and functional data, raising specific challenges. Our research group is participating in an example of successful de-identified data sharing: the Transforming Research and Clinical Knowledge for Traumatic Brain Injury (TRACK-TBI), a series of two large-scale prospective multicenter observational trials for improving traumatic brain injury diagnosis and therapeutic targeting (ClinicalTrials.gov Identifiers NCT01565551, pilot 2010–13; NCT02119182, ongoing 2014–18) (Yue et al. 2013). The pilot phase consisted of three centers and collected data on 599 patients. The TRACK-TBI project applies the official NIH/NINDS TBI Common Data Elements (TBI-CDEs) and standardized collection protocols for biospecimens (Diaz-Arrastia et al. 2014), imaging (Yuh et al. 2013; 2014), and neurocognitive and neuropsychiatric outcome metrics (Dams-O'Connor et al. 2013; Lingsma et al. 2014).

Multicenter patient data, including protected health information (PHI) as defined by HIPAA, is collected under informed consent into a central, custom-designed repository (QuesGen Systems, Inc., Burlingame, CA) that assigns a globally unique identifier. The system manages permissions ranging from 'no-PHI', to 'local PHI only', to 'full-access'. The 'full-access' view is only available to a small quality control and assurance team. Investigators have access to only local PHI from their enrolling center, and only if access is pre-approved by the local institutional review board. Multicenter data are broadly searchable in the no-PHI view by the full group of TRACK-TBI investigators. Ultimately, portions of the 'no-PHI' view of TRACK-TBI data will be publicly accessible for research purposes through the US Federal Interagency TBI Research (FITBIR) informatics system (https://fitbir.nih.gov). Additionally, data use agreements guide the handling of data, and subject timeline data are recoded from dates to days from baseline. These processes

have been expanded upon in the current TRACK-TBI study funded for 11 centers with goals of capturing data on over 3000 patients in the next 4 years.

Privacy Concerns and Use

Patients and research subjects have long expressed concern about privacy of health information. In a study by King et al. (2012), participants preferred to be asked for permission before their health information was used for any purpose other than medical treatment (92 %), and they wanted to know the details of the research before allowing the use of their health records (83 %). The study showed that there are some particularly sensitive issues, including family medical history, genetic disorders, mental illness, drug or alcohol related incidents, lists of previous procedures, and current medications. There are also ethical doubts about the ability of cognitively impaired subjects to give informed consent or addicted subjects to participate in studies that involve the administration of drugs of dependence. Tests to identify addicts or predict risk of addiction will raise concerns about invasion of privacy, third-party use of data, and the powers of courts to coerce defendants to undergo such tests (Hall et al. 2004).

Privacy around genetic data has also become a concern among researchers. Lapses in data security can result in undesired publicity and expense (Benitez and Malin 2010). Kho et al. (2009) write that "to ensure that legislation on privacy does not unduly bias observational studies using medical records, thoughtful decision making by research ethics boards on the need for mandatory consent is necessary". Again, attitudes and practices vary widely. In interviews with administrators at Canadian universities, 47 % of sites required individual patient consent for studies to proceed, but 45 % did not require consent or suggested a notification and opt-out process (Willison et al. 2008). Lane and Schur (2010) proposed that a guiding research principle should be "to generate released data that are as close to the maximum acceptable risk as possible. HIPAA and other privacy measures can perhaps be seen as having had the effect of lowering the 'maximum acceptable risk' level and rendering some data unreleasable."

Going Forward

The ability to integrate clinical, genetic, imaging, and other types of biomedical data will be of tremendous value in ongoing efforts to discover and develop biomarkers and drugs to address unmet medical needs. New technologies and processes can simultaneously support data privacy, data sharing, and research objectives. Here, we described several basic principles as well as experiences from our research group to illustrate that data privacy and sharing can be accomplished together. Our experience highlights an approach in the context of an ambitious basic and clinical research collaboration.

Van Horn and Ball (2008) write that although it will not be "a pain-free process, with increased data availability, scientists from multiple fields can enjoy greater opportunity for novel discoveries about the brain in health and disease." Despite the rapid pace of technological change, it will be critical to think carefully about balancing concerns and objectives. Neuroscientists and neurologists, as well as regulators and patients, can seek opportunities to collaborate with research and clinical colleagues as they advocate for data privacy and high quality science and medicine.

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Circulating Brain-Derived Neurotrophic Factor Has Diagnostic and Prognostic Value in Traumatic Brain Injury

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Abstract

Brain-derived neurotrophic factor (BDNF) is important for neuronal survival and regeneration. We investigated the diagnostic and prognostic values of serum BDNF in traumatic brain injury (TBI). We examined serum BDNF in two independent cohorts of TBI cases presenting to the emergency departments (EDs) of the Johns Hopkins Hospital (JHH; $n = 76$) and San Francisco General Hospital (SFGH, $n = 80$), and a control group of JHH ED patients without TBI ($n = 150$). Findings were subsequently validated in the prospective, multi-center Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) Pilot study ($n = 159$). We investigated the association between BDNF, glial fibrillary acidic protein (GFAP), and ubiquitin C-terminal hydrolase-L1 (UCH-L1) and recovery from TBI at 6 months in the TRACK-TBI Pilot cohort. Incomplete recovery was defined as having either post-concussive syndrome or a Glasgow Outcome Scale Extended score <8 at 6 months. Median day-of-injury BDNF concentrations (ng/mL) were lower among TBI cases (JHH TBI, 17.5 and SFGH TBI, 13.8) than in JHH controls (60.3; $p = 0.0001$). Among TRACK-TBI Pilot subjects, median BDNF concentrations (ng/mL) were higher in mild (8.3) than in moderate (4.3) or severe TBI (4.0; $p = 0.004$. In the TRACK-TBI cohort, the 75 (71.4%) subjects with very low BDNF values (i.e., <the 1st percentile for non-TBI controls, <14.2 ng/mL) had higher odds of incomplete recovery than those who did not have very low values (odds ratio, 4.0; 95% confidence interval [CI]: 1.5-11.0). The area under the receiver operator curve for discriminating complete and incomplete recovery was 0.65 (95% CI: 0.52-0.78) for BDNF, 0.61 (95% CI: 0.49-0.73) for GFAP, and 0.55 (95% CI: 0.43-0.66) for UCH-L1. The addition of GFAP/UCH-L1 to BDNF did not improve outcome prediction significantly. Day-of-injury serum BDNF is associated with TBI diagnosis and also provides 6-month prognostic information regarding recovery from TBI. Thus, day-of-injury BDNF values may aid in TBI risk stratification.

Key words: biomarkers; brain-derived neurotrophic factor; glial fibrillary acidic protein; traumatic brain injury; ubiquitin C-terminal hydrolase-L1

Introduction

DIAGNOSIS OF TRAUMATIC BRAIN INJURY (TBI) and early identification of patients at risk for long-term consequences of TBI represents a unique clinical challenge with major public health

implications. A number of candidate circulating TBI biomarkers have shown promise for aiding in the diagnosis of TBI and in identifying patients with traumatic abnormalities on head computed tomography (CT) scan.[1–4] Importantly, their ability to predict adverse consequences of TBI has been limited. Objective diagnosis

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and prognosis of TBI will help improve triaging to appropriate medical care at time of injury, guide judicious use of neuroimaging, and inform the development of ''return to work or play'' guidelines. Additionally, while most patients with mild TBI (mTBI) recover to their pre-injury state within 3 months, a significant minority do not. Prognostic biomarkers that identify patients unlikely to make a full recovery are needed to identify appropriate candidates for clinical trials of novel TBI therapies.[5]

Brain-derived neurotrophic factor (BDNF), a member of the family of neurotrophic proteins, is a secreted autocrine factor that promotes the development, maintenance, survival, differentiation, and regeneration of neurons.[6,7] It is also important for synaptic plasticity and memory processing.[8,9] BDNF has been implicated in reducing secondary brain injury, with elevations providing neuro-protection and restoring connectivity after TBI.[10–12] However, the diagnostic and prognostic significance of day-of-injury circulating BDNF concentration are not well understood. We conducted a study to establish the association between BDNF and TBI and to determine whether day-of-injury BDNF values are associated with TBI severity and outcomes.

Glial fibrillary acidic protein (GFAP) is an astrocytic protein whose functions include cell communication, mitosis, and main-taining the integrity of the blood–brain barrier (BBB).[13] GFAP has excellent specificity for TBI-associated intracranial hemorrhage and focal mass lesions.[14,15] Elevated values are associated with increased mortality.[1,16]

Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is a neuronal protein that is involved in the addition and removal of ubiquitin proteins flagged for metabolism. UCH-L1 is especially elevated in TBI and has been found to be associated with mortality.[17–19]

Methods

BDNF serum concentrations were determined in duplicate in two independent cohorts of TBI cases presenting to the Johns Hopkins Hospital (JHH) and the San Francisco General Hospital (SFGH) emergency departments (ED), and one control cohort of JHH ED patients presenting for non-TBI complaints. Findings were subsequently validated in the prospective, multi-center Transforming Research and Clinical Knowledge in TBI Pilot (TRACK-TBI) Pilot study.[20] We also compared the prognostic value of BDNF to that of two well-studied TBI biomarkers, GFAP and UCH-L1, since these biomarker values were available from a previous study.[18] Study protocols were approved by the institutional review boards at participating sites.

Study population

Case cohorts.   JHH and SFGH are academic, tertiary care, Level 1 trauma institutions. Patients were eligible for inclusion as TBI cases if they presented to JHH and SFGH ED after experiencing acute blunt head trauma and met the following criteria: age 18 years or older; presented within 24 h of injury; met the American College of Emergency Physicians (ACEP) criteria for obtaining head CT scans in TBI[21]; received a non-contrast head CT scan as part of their clinical evaluation; and had excess serum sample available in the clinical chemistry lab. Cases met the definition of TBI proposed by the Demographics and Clinical Assessment Working Group of the International and Interagency Initiative toward Common Data Ele-ments (TBI CDE) for Research on Traumatic Brain Injury and Psychological Health.[22] Eligible cases were excluded if they had one of the following prior medical conditions: demyelinating disease, neurodegenerative disease, dementia, stroke, brain tumor, intracra-nial surgery, or active cancer. TBI cases were selected between November 2012 and September 2013. Since we utilized excess clinical blood samples, informed consent was waived.

Control cohort.   Patients included as control subjects were JHH ED patients who were evaluated for suspected acute coro-nary syndrome,[23] had no blunt head trauma in the preceding 7 days, and were deemed to have a non-cardiac condition and discharged home from the ED. Eligible control subjects were excluded if they met any of the exclusion criteria for cases (see above). Control subjects did not receive head CT scans since there was no clinical indication for doing so. Clinical and demographic data were collected via structured patient interviews and a review of the electronic medical record. Subjects were enrolled between January 2012 and February 2013. Written informed consent was obtained from all subjects.

Validation cohort.   The TRACK-TBI Pilot study enrolled subjects 16 years and older who presented to SFGH ED, the Uni-versity of Pittsburgh Medical Center (UPMC) ED, and the Uni-versity Medical Center Brackenridge (UMCB), Austin, TX, ED with TBI.[20] Patients were included in the study if they presented to the ED within 24 h of acute blunt force head trauma and met the ACEP criteria for obtaining a head CT in TBI, as previously de-scribed.[20] Only subjects from TRACK-TBI Pilot who had serum samples available for testing were included in the present study. Subjects in the validation cohort were enrolled from April 2010 to June 2011, and were distinct from those in the SFGH case cohort. Written informed consent was obtained from all subjects prior to enrollment in the study. Subjects unable to provide consent due to their injury were consented through their legally authorized rep-resentative at time of injury, and re-consented if cognitively able during their inpatient stay and/or their follow-up assessment time-point.

Serum sample collection and biomarker measurement

For the JHH and SFGH TBI case cohorts, excess serum samples stored in a 4°C refrigerator were retrieved from their respective clinical chemistry laboratory and stored in a -80°C freezer. These samples were kept at 4°C for variable duration (median of 5 days). Serum samples for JHH control subjects and for TRACK-TBI Pilot subjects were collected, processed and stored in a -80°C freezer within 2 h of collection, as previously described.[23,24] Samples for TRACK-TBI patients were collected within 24 h of injury.[13]

Samples were randomized and BDNF assayed in batches with an electrochemiluminescent sandwich immunoassay and read with a Sector Imager 2400 (Meso Scale Discovery, Rockville, MD). BDNF assay capture (MAB848) and detection antibodies (MAB648) and assay standard (248BD005) were obtained from R&D Systems (Duoset reagents, Cat. # DY248; Minneapolis, MN). Assays were performed within a single laboratory by staff blinded to clinical outcomes or study cohort. Samples from the different cohorts were shipped to this single academic laboratory. The assay lower limit of detection (LOD) was 0.0125 ng/mL and the lower limit of quantification was 0.5 ng/mL. As specified by the manu-facturer, these assay reagents have no overlap with the TrK receptor proteins B-NGF, GDNF, NT-3, and NT-4. Assays were performed in duplicate. A previous study examining the stability of BDNF in blood samples stored at room temperature for 0-24 h, 24-48 h, 48-72 h, or >72 h revealed an average increase of 1.67 (95% CI: 1.08-2.26) ng/mL per each 24-h period.[25] Since BDNF values are high in healthy subjects and low in diseased subjects, we defined low BDNF values as values that are lower than the 1st percentile in JHH non-TBI control subjects. This is analogous to the use of the 99th percentile as the recommended cut-off value in cardiac biomark-ers[26,27] (values are high in diseased and low in healthy subjects).

GFAP and UCH-L1 were previously measured in TRACK-TBI Pilot in a single laboratory (Banyan Biomarkers, Alachua, FL).[15,18] The LOD of GFAP and UCH-L1 were 0.1 ng/mL and 0.03 ng/mL, respectively.

TABLE 1. THE DEPARTMENT OF DEFENSE/DEPARTMENT OF VETERANS AFFAIRS CLASSIFICATION OF TBI SEVERITY

Criteria	Mild	Moderate	Severe
Head CT/MRI	Normal	Normal/ abnormal	Normal/ abnormal
Loss of consciousness	0-30 min	>30 min and <24 h	>24 h
Alteration of consciousness/mental state	<24 h	>24 h	>24 h
Post-traumatic amnesia	<1 day	>1 and <7 days	>7 days
Best Glasgow Coma Scale score within first 24 h	13–15	9–12	<9

TBI, traumatic brain injury; CT, computed tomography; MRI, magnetic resonance imaging.

Outcomes

All patients enrolled in TRACK-TBI Pilot received head CT scans at the time of presentation to the ED. Each head CT was de-identified and read by a blinded board-certified neuroradiologist following the recommendations of the TBI-CDE Neuroimaging Working Group.[28] Our primary outcome, incomplete recovery at 6 months, was defined as a composite outcome of either post-concussive syndrome (PCS) or Glasgow Outcome Scale Extended (GOSE) score of <8 at 6 months, as these two measures together encompass a wider spectrum of the entire sphere of post-TBI outcomes. We defined PCS as having three or more symptoms on the 6-month Rivermead Post-Concussion Questionnaire[29] that were rated as worse than before the injury (score of 2).[30] The GOSE categorizes recovery after TBI on a scale of 1–8, where 1=dead and 8=upper good recovery. GOSE <8 signifies incomplete recovery.[31] Additionally, head CT findings were classified as traumatic lesion present (this does not include isolated skull fractures) or no traumatic lesion present. TBI severity was classified as mild, moderate, or severe based on the Department of Defense/Department of Veterans Affairs definition (Table 1).[32]

Statistical analyses

Clinical and demographic data were summarized with descriptive statistics and differences were examined using the Mann-Whitney test (2-groups), the Kruskal-Wallis test (n-groups) and the χ^2 test (proportions). We quantified the discriminative ability of BDNF to distinguish between cases and controls, and to distinguish between TBI patients with relevant clinical outcomes and those without using area under the receiver operator curve (AUC). We also constructed logistic regression models to evaluate the association between BDNF values and clinical outcomes. We compared the AUCs of combinations of BDNF, GFAP, and UCH-L1 for discriminating between relevant clinical outcomes, using the method suggested by DeLong and colleagues.[33] This is a widely cited and generally accepted method that provides the confidence interval and standard error of the difference between two (or more) correlated AUCs.

To understand the determinants of BDNF in the control population, we constructed univariable and multi-variable linear regression models. Variables included in the models (age, gender, race, blood pressure, history of hypertension, history of depression or schizophrenia)[34–37] were selected based on an *a priori* literature

TABLE 2. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF STUDY POPULATION

	JHH non-TBI controls n=150	JHH TBI cases n=76	SFGH TBI cases n=76	TRACK-TBI pilot cases n=159	p value
Median age in years (IQR)	54 (47 – 62)	47 (30 – 56)	42 (26 – 56)	41 (25 – 56)	<0.001
Female (%)	79 (52.7)	29 (38.2)	22 (29.0)	45 (28.3)	<0.001
Race (%)					<0.001
• African-American	116 (77.3)	41 (54.0)	5 (6.6)	15 (9.5)	
• White	30 (20.0)	25 (32.9)	59 (77.6)	132 (83.5)	
• Other	4 (2.7)	10 (13.2)	12 (15.8)	11 (7.0)	
Mechanism of injury (%)					0.003
• Assault		19 (25.0)	13 (17.1)	23 (14.6)	
• Fall		26 (34.2)	23 (30.3)	50 (31.6)	
• MVC		21 (27.6)	11 (14.5)	51 (32.3)	
• Pedestrian struck		4 (5.3)	14 (18.4)	9 (5.7)	
• Struck by/against		3 (4.0)	2 (2.6)	5 (3.2)	
• Other trauma		3 (4.0)	13 (17.1)	20 (12.7)	
Glasgow Coma Scale (%)					0.09
• 3-8		5 (6.6)	4 (5.4)	19 (12.0)	
• 9-12		3 (4.0)	4 (5.4)	6 (3.8)	
• 13		2 (2.6)	3 (4.0)	1 (0.6)	
• 14		11 (14.5)	20 (27.0)	22 (13.8)	
• 15		55 (72.4)	43 (58.1)	111 (69.8)	
Traumatic intracranial abnormality on head CT (%)		21 (27.6)	24 (31.6)	75 (47.2)	0.006

JHH, Johns Hopkins Hospital; TBI, traumatic brain injury; SFGH, San Francisco General Hospital; TRACK-TBI, Transforming Research and Clinical Knowledge in TBI study; IQR, interquartile range; MVC, motor vehicle collision ; CT, computed tomography.
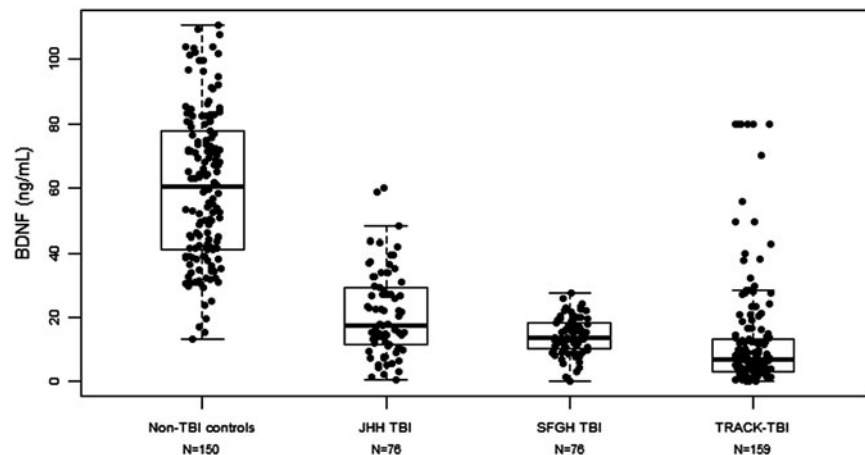
FIG. 1. Distribution of brain-derived neurotrophic factor (BDNF) in traumatic brain injury (TBI) and non-TBI cohorts. Graphical distribution of individual BDNF values and the corresponding box plots for Johns Hopkins Hospital (JHH) non-TBI control subjects, JHH TBI cases, San Francisco General Hospital (SFGH) TBI cases and Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) Pilot cases.

review. A two-tailed *p* value of < 0.05 was considered statistically significant. Statistical analyses were performed using STATA/MP statistical software version 11.2 (StataCorp, College Station, TX), and RStudio statistical software version 0.97.312 (Boston, MA).

Results

A total of 311 TBI cases were analyzed: 76 cases in the JHH TBI cohort, 76 cases in the SFGH TBI cohort, and 159 cases in TRACK-TBI Pilot, in addition to 150 JHH non-trauma control subjects. Non-trauma control subjects were older and more likely to be female or African-American, compared with TBI cases (Table 2).

Association between BDNF and TBI

In the initial case-control study, median day-of-injury BDNF values (ng/mL) were lower among TBI cases (17.5; interquartile range [IQR], 11.3-29.6) in JHH TBI group and 13.8 (IQR, 10.1-18.3) in the SFGH group) than in non-TBI controls (60.3; IQR, 41.1-78.2; *p* = 0.0001). The 1st percentile of BDNF values in JHH non-TBI controls was 14.2 ng/mL. BDNF discriminated between TBI cases (JHH and SFGH) and non-TBI controls with an AUC of 0.96 (95% CI: 0.94-0.98), which is considered excellent accuracy. There was no significant association between duration of storage of serum samples in 4°C and BDNF value among TBI cases (Supplementary Fig. 1; see online supplementary material at www.liebertpub.com). Similarly, in a validation study, median day-of-injury BDNF values (ng/mL) were found to be low among TRACK-TBI Pilot subjects (6.8; IQR, 3.0-13.5). The distribution of BDNF values among the TBI cases and non-TBI control subjects studied is presented in Figure 1. BDNF discriminated between TRACK-TBI Pilot cases and JHH non-TBI controls with an AUC of 0.94 (95% CI: 0.91-0.97; Fig. 2). BDNF values were lower in TRACK-TBI Pilot cases (prospectively collected samples) than in the JHH or SFGH cohorts (excess clinical samples; *p* < 0.001). BDNF discriminated between JHH non-TBI controls and TRACK-TBI cases classified as mild TBI with an AUC of 0.95 (95% CI: 0.92-0.98).

*Association between BDNF and TBI severity*

Within the TRACK-TBI Pilot cohort, day-of-injury BDNF values (ng/mL) were higher in mild TBI subjects (8.3; IQR, 5.2-

16.5) than in moderate (4.3; IQR, 1.8-10.1) or severe TBI (4.0; IQR, 1.5-13.8; *p* = 0.003). The JHH and SFGH cohorts did not have sufficient moderate and severe TBI patients to assess BDNF variation with TBI severity (Table 1). Among TRACK-TBI Pilot subjects, median day-of-injury BDNF values (ng/mL) were higher in subjects without intracranial abnormality on head CT (8.4; IQR, 5.2-16.6) than in subjects with intracranial abnormality on head CT (4.2; IQR, 1.8-10.1; *p* < 0.001; Fig. 3). BDNF discriminated between subjects with and without intracranial abnormality on head CT with an AUC of 0.67 (95% CI: 0.58-0.75). In the JHH cohort, median BDNF (ng/mL) for normal CT and abnormal head CT were 17.8 (IQR, 12.5-30.8) and 16.2 (IQR, 4.8-23.2), respectively (*p* = 0.13). Whereas in the SFGH cohort, median BDNF (ng/mL) for normal and abnormal head CT were 13.0 (IQR, 9.4-17.1) and 15.1 (IQR, 10.5-21.3), respectively (*p* = 0.17.

Association between BDNF and TBI outcomes

Among the 159 TRACK-TBI Pilot subjects, 94 (59%) had the Rivermead Post-Concussion Questionnaire measured and 111 (69%) had the GOSE score measured at 6 months post-injury. Of those with 6-month outcome measures, 62% (58/94) were determined to have PCS, 70% (78/111) had a GOSE < 8, and 80% (85/106) had either PCS or GOSE < 8. Among the 94 subjects with both PCS and GOSE measures, 51 (54%) had both PCS and GOSE < 8, 21 (22%) had neither PCS nor GOSE < 8, 15 (16%) had GOSE < 8 but no PCS, and seven (7%) had PCS and GOSE = 8. Day-of-injury BDNF values (ng/mL) were not significantly different between subjects with PCS (7.2; IQR, 3.0-12.8) and those without PCS (7.1; IQR, 4.0-21.0), or between subjects with GOSE = 8 (7.9; IQR, 4.0-23.3) and those with GOSE < 8 (7.1; IQR, 2.8-13.0). The 76 (72.4%) TRACK-TBI subjects who had very low BDNF values (i.e., less than the 1st percentile for non-TBI controls [< 14.2 ng/mL]) had higher odds of incomplete recovery than those without very low BDNF (odds ratio, 4.0; 95% CI: 1.5-11.0). Very low BDNF values were associated with higher odds of incomplete recovery among those with mild TBI (4.9; 95% CI: 1.3-17.9) than those with moderate or severe TBI (2.0; 95% CI: 0.3-12.5).

There was a trend toward higher BDNF values as the time interval between injury and serum sampling for BDNF measurement increased (Fig. 4). The trend was similar among those with
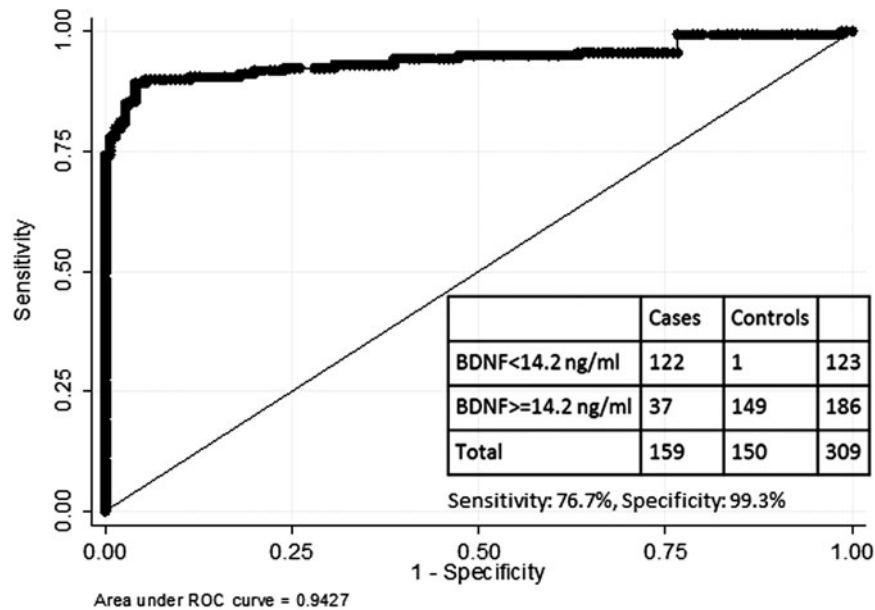
FIG. 2. Receiver operator curve for distinguishing between traumatic brain injury (TBI) cases and controls with brain-derived neurotrophic factor (BDNF). The receiver operator curve for discriminating between Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) Pilot cases and Johns Hopkins Hospital (JHH) controls using BDNF values. The table reports the diagnostic accuracy of using a cut-off value of 14.2 ng/mL for distinguishing between TBI cases and controls.

complete and incomplete recovery. However, this trend did not reach statistical significance ($p = 0.10$). Similarly, there was a trend toward lower BDNF values with increasing age (Fig. 5). However, this trend was not statistically significant ($p = 0.09$). After adjustment for age and time between injury and serum sampling for BDNF measurement, very low BDNF (<14.2 ng/mL) remained statistically significantly associated with incomplete recovery (odds ratio, 4.16; 95% CI: 1.48-11.70).

Performance of GFAP and UCH-L1, compared with BDNF

A comparison of TRACK-TBI Pilot GFAP and UCH-L1 values with BDNF assayed on the same samples showed that GFAP, BDNF and UCH-L1 discriminated between subjects with traumatic abnormalities on head CT and those without, with AUCs of 0.88 (95% CI: 0.83-0.93) for GFAP, 0.70 (95% CI: 0.62-0.79) for UCH-L1, and 0.67 (95% CI: 0.58-0.75) for BDNF. They also discriminated between subjects with complete recovery from TBI and those without, with AUCs of 0.65 (95% CI: 0.52-0.78) for BDNF, 0.61 (95% CI: 0.49-0.73) for GFAP, and 0.55 (95% CI: 0.43-0.66) for UCH-L1 at 6 months. A comparison of the discriminative abilities of the biomarkers examined is presented in Table 3. There was no minimal correlation between BDNF and GFAP values ($r = -0.11$; $p = 0.16$) and between BDNF and UCH-L1 values ($r = 0.07$; $p = 0.36$), suggesting that they may be associated with different pathways of injury. To determine whether combining biomarkers resulted in improved discrimination of complete versus incomplete recovery, we used combinations of two biomarkers, instead of all three biomarkers, since only 21 subjects had complete recovery (10 events per predictor variable is required for adequate statistical power).[38] Addition of GFAP to BDNF did not improve the discrimination of complete versus incomplete recovery (AUC was 0.66 instead of 0.65; $p = 0.76$). Similarly, addition of UCH-L1 to

BDNF did not improve the discrimination of complete versus incomplete recovery (AUC was 0.66 instead of 0.65; $p = 0.55$).

Predictors of BDNF values in non-TBI control subjects

Among non-TBI controls, after adjustment for age, gender, race, hypertension, diabetes, history of psychiatric illness, and mean arterial pressure, only gender and mean arterial pressure remained independent predictors of BDNF among non-TBI controls (Table 4). Median BDNF levels (ng/mL) were greater in females (69.1; IQR, 41.4-82.4; $n = 79$) than in males (52.7; IQR, 38.7-71.8; $n = 71$; $p = 0.049$). However, there were no gender differences in BDNF levels within the TBI cohorts examined. Among non-TBI controls, BDNF values increased with increasing mean arterial pressure. However, there was no statistically significant association between BDNF and blood pressure within the TBI cohorts examined.

Discussion

We report the diagnostic value of day-of-injury circulating BDNF for TBI, and its ability to be prognostic for identifying subjects likely to have persistent TBI-related sequelae at 6 months. Further, we have determined that BDNF has a higher prognostic value among mild TBI subjects than moderate/severe TBI subjects. The dysregulation of BDNF in TBI has been examined with equivocal findings by a number of studies using animal models of TBI.[10] In the majority of these studies, BDNF mRNA expression was measured in brain tissue, with reports of upregulation of BDNF mRNA in the hippocampus and cerebral cortex.[39–41] However, other studies have suggested reduced secretion of brain BDNF protein after TBI, with subsequent increased secretion following experimental TBI treatment.[42] Few studies have measured circulating BDNF in human TBI subjects. Two small pediatric studies reported no differences in plasma BDNF levels between human
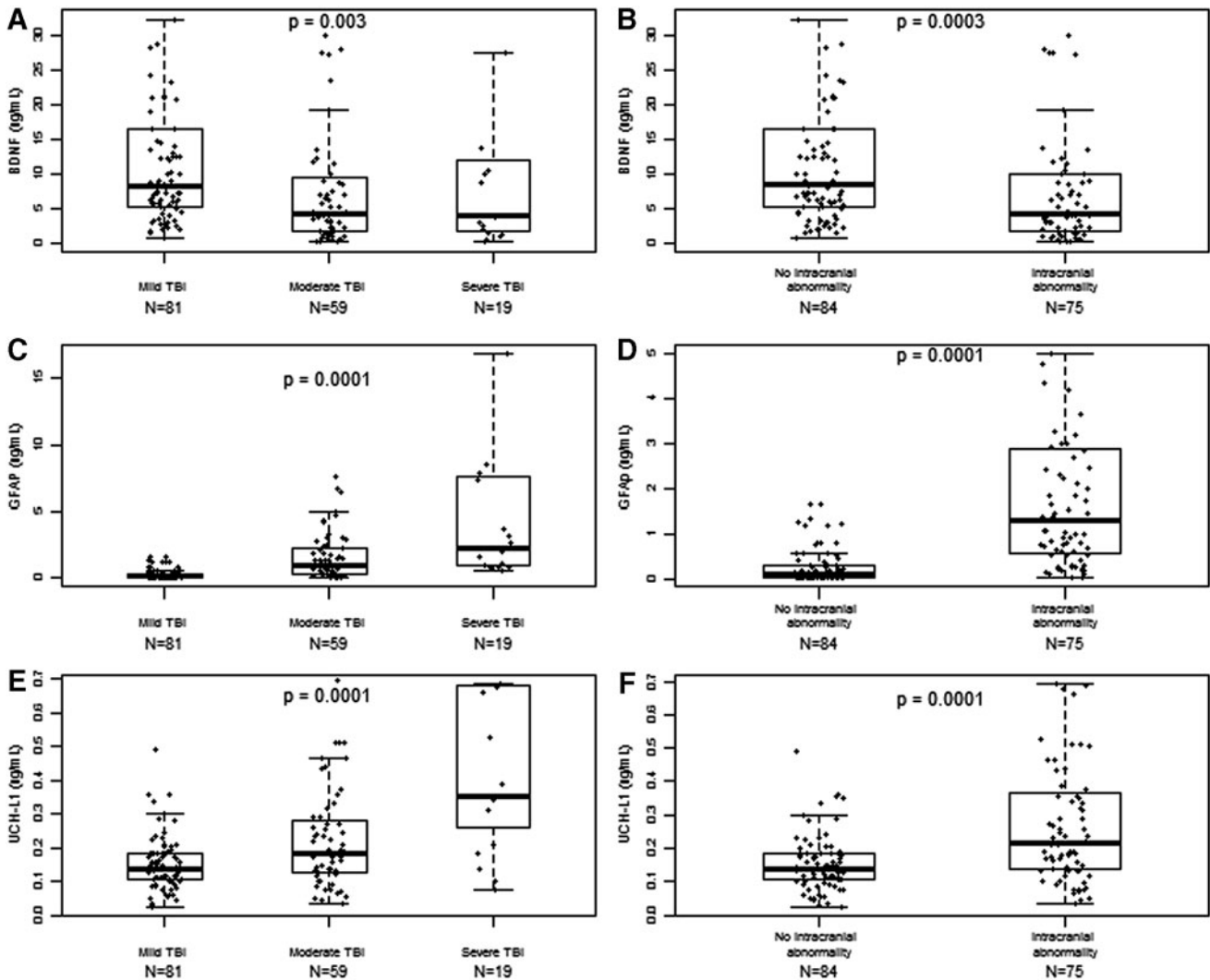
FIG. 3. Association between biomarkers examined and traumatic brain injury (TBI) severity. Presented are the graphical distribution of individual brain-derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP), and ubiquitin C-terminal hydrolase-L1 (UCH-L1) values in Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) Pilot and the corresponding boxplots according to TBI severity, classified as mild, moderate, or severe; and the presence or absence of traumatic intracranial abnormality on head computed tomography (CT) scan: **(A)** depicts BDNF versus TBI severity classified as mild moderate or severe; **(B)** depicts BDNF versus TBI severity classified by CT scan; **(C)** depicts GFAP versus TBI severity classified as mild, moderate, or severe; **(D)** depicts GFAP versus TBI severity classified by head CT scan; **(E)** depicts UCH-L1 versus TBI severity classified as mild, moderate or severe; **(F)** depicts UCH-L1 versus TBI severity classified by head CT scan. Individual values that were extreme outliers are excluded from the graphical presentation.

TBI cases and non-trauma controls.[43,44] However, control subjects in these studies had abnormal neurologic status (obstructive hydrocephalus undergoing elective surgery,[43] and subjects undergoing lumbar puncture for suspected meningitis[44]).

Another study measuring BDNF in Olympic boxers and healthy controls also reported no differences in plasma BDNF.[45] However this study measured BDNF in plasma samples obtained 1–6 days after a bout and the release and clearance kinetics of BDNF in humans is not known. Further, Buonora and colleagues recently reported higher plasma BDNF levels in TBI cases, compared with controls.[46] Our findings and study design are most similar to results reported by Kalish and Phillips.[47] These investigators measured BDNF in serum samples obtained from 30 TBI patients and reported decreasing BDNF with increasing severity of TBI. Our study has demonstrated in three separate TBI cohorts that circulating

levels of BDNF are lower in TBI cases, compared with non-trauma controls.

BDNF is limited in its ability to distinguish between TBI subjects with and without intracranial abnormalities. This may be due to the fact that structural proteins (such as GFAP) are more likely to have a strong association with radiographic changes in TBI than secreted proteins. However, secreted proteins may reflect both primary and secondary brain injury and therefore may have a stronger association with long-term outcomes. Our findings demonstrate that BDNF has higher prognostic value in mTBI subjects, compared with moderate or severe TBI patients. Therefore, BDNF holds promise for improving clinical prognostication of outcomes in TBI patients who have no intracranial abnormalities on head CT scans.

BDNF is an appealing candidate biomarker for detecting TBI for numerous reasons. First, our study results demonstrate a very strong
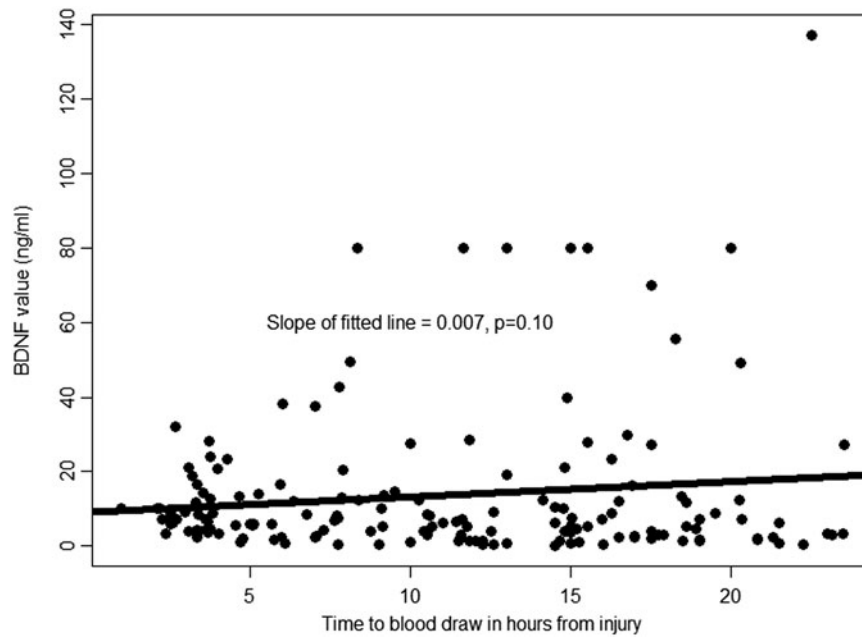
FIG. 4. Association between brain-derived neurotrophic factor (BDNF) and time from injury to blood sampling. This is a scatter plot of the association between day-of-injury BDNF values and time between injury and blood draw (in hours). The line represents the best fitting linear regression line that summarizes this association.

association between BDNF and TBI, yielding excellent discriminative ability of 0.94-0.95 (as measured by the c-statistic). Second, our findings were replicated across three different TBI cohorts. Third, we have demonstrated an association between BDNF and TBI severity and an association between BDNF and TBI outcome. Finally, the association between TBI and BDNF is biologically plausible and has been demonstrated in diverse TBI models including animal models.[10]

BDNF is the most abundantly expressed brain neurotrophin[48] and as a secreted protein, can be readily and reliably measured in serum using well established immuno-assay techniques, identifying it as a non-necrosis brain injury biomarker. This distinguishes BDNF from other protein-based biomarkers that are structural components of neurons and glial cells—for example, GFAP (an astro-glial intermediate filament cytoskeletal protein), S100B (an intracellular calcium binding protein), UCHL1 (a ubiquitin ligase
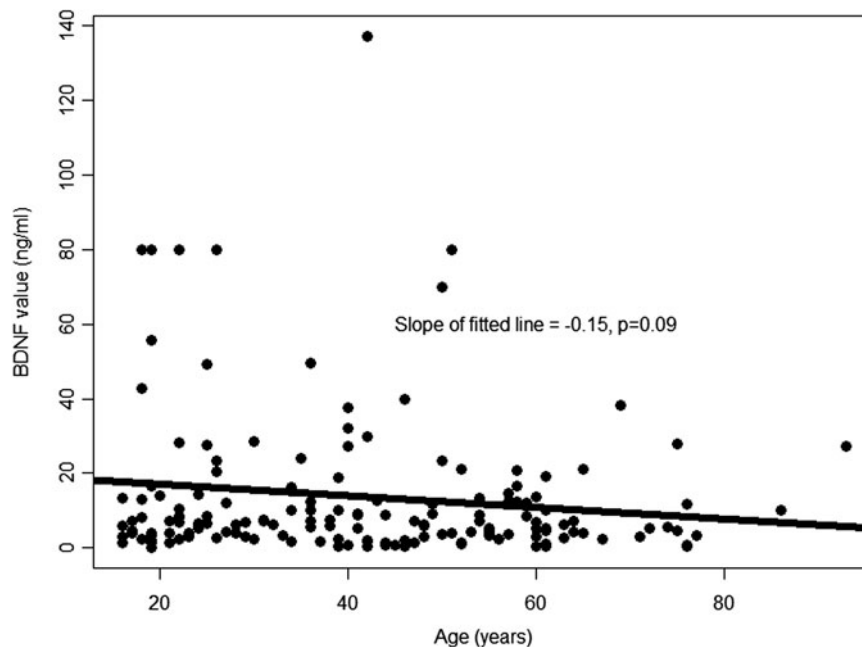
FIG. 5. Association between brain-derived neurotrophic factor (BDNF) and age. This is a scatter plot of the association between day-of-injury BDNF values and age (in years). The line represents the best fitting linear regression line that summarizes this association.

TABLE 3. DISCRIMINATIVE ABILITY OF DIFFERENT BIOMARKERS FOR RELEVANT TBI OUTCOMES AS MEASURED BY THE AREA
UNDER THE RECEIVER OPERATOR CURVE (AUC) AND THE CORRESPONDING 95% CONFIDENCE INTERVAL

Outcome	GFAP	UCH-L1	BDNF
GOSE score <8	0.61 (0.50-0.71)	0.55 (0.44-0.66)	0.56 (0.44-0.68)
Post-concussive syndrome (PCS)	0.56 (0.44-0.68)	0.52 (0.40-0.64)	0.55 (0.43-0.68)
Composite (GOSE score <8 or PCS)	0.61 (0.49-0.73)	0.55 (0.43-0.66)	0.65 (0.52-0.78)
Intracranial abnormality on head CT	0.88 (0.83-0.93)	0.70 (0.62-0.79)	0.67 (0.58-0.75)

TBI, traumatic brain injury; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; BDNF, brain-derived neurotrophic factor; GOSE, Glasgow Outcome Scale Extended; CT, computed tomography.

localized to the neuronal soma), neurofilaments (cytoskeletal components of axons), cleaved tau (intracellular microtubule-associated proteins), and myelin basic proteins (a component of myelin), among others.[49] In order for structural proteins to be found in high abundance in circulation, sufficient cellular necrosis and damage to the BBB is required. However, BDNF does not require cellular necrosis or damage to the BBB to be observed in circulation.[50] Further, this allows BDNF to be more abundant in circulation than structural proteins, increasing assay sensitivity.

The exact mechanisms underlining the dysregulation of BDNF in TBI are not yet well understood. Although some studies implicate BDNF in neuroprotection following injuries,[51,52] other studies suggest it contributes to neurodegenerative events that occur following injury.[53,54] It also has been suggested that BDNF ameliorates the impact of secondary brain damage by modifying BDNF-induced gene expression.[10] Following TBI and acute disconnection of brain circuitry, there is an attempt at reorganization and reconnection of brain circuits. BDNF promotes synaptic plasticity and restoration during the brain circuitry "reconnection" phase. We have found that post-TBI BDNF levels behave unlike the majority of candidate biomarkers of TBI. Lower BDNF values are associated with worse prognosis, whereas with other TBI biomarkers, lower values are typically associated with better prognosis,[4] with the exception of microtubule-associated protein 2, a dendritic marker, which has higher values at 6 months after injury in severe TBI subjects with improved outcomes.[3] We postulate that during the acute phase of TBI, the formation of new neuronal circuits might not be advisable, and therefore there may be no need for increased production of neurotrophic factors. However, it is possible that the initial decrease in circulating BDNF during the acute phase of trauma (as seen in our study) is potentially followed by a subsequent increase, especially during the sub-acute/chronic phases of TBI. Understanding the temporal variations in BDNF expression will be an important first step towards further elucidating the biological functions of BDNF in TBI and recovery. It is also possible that since decreased BDNF levels are found in patients with anxiety,[55] major depressive disorder,[56] and schizophrenia,[57] low BDNF values on the day of injury identifies subjects at risk for these conditions (whether previously recognized or otherwise) and predisposes this population to incomplete recovery.

Although circulating BDNF may originate from the hippocampus, cerebral cortex, and basal forebrain,[58] it also may be derived from other cellular sources, including platelets,[59,60] smooth muscle cells,[35,61] and vascular endothelial cells.[62] This supports BDNF's role as a promoter of neuronal growth and survival both in the central and peripheral nervous system. However, it is unclear whether circulating BDNF values measured in this study are representative of central nervous system values. Prior studies suggest that BDNF crosses the BBB bi-directionally.[63] Further, it has been reported that serum and cortical BDNF values are strongly correlated.[64] Irrespective of the exact source(s) of circulating BDNF, our finding that circulating BDNF values are suppressed in TBI and that low BDNF values are associated with poor recovery suggest that BDNF deserves further evaluation as a potential biomarker of TBI and TBI recovery.

BDNF has the potential to become a surrogate marker of successful TBI treatment. In a study examining dietary omega-3 fatty acid supplementation in TBI, rats with decreased brain BDNF following mild fluid percussion injury had normalized brain BDNF levels and improved learning ability following 4 weeks of dietary supplementation with omega-3 fatty acids.[42] Similarly, rats exposed to delayed exercise (2–3 weeks after injury) had increases in

TABLE 4. DETERMINANTS OF BDNF IN THE CONTROL POPULATION (N = 150)

	Unadjusted regression co-efficient (95% CI)	Adjusted regression co-efficient (95% CI)	p Value for adjusted regression co-efficient
Age in years	-0.1 (-0.4 to 0.3)	-0.2 (-0.5 to 0.2)	0.38
Gender			
• Female	Reference	Reference	0.04
• Male	-7.2 (-15.4 to 1.0)	-8.8 (-17.0 to -0.6)	
Race			
• African-American	Reference	Reference	
• Caucasian	-9.6 (-19.8 to 0.7)	-8.8 (-18.8 to 1.3)	0.09
• Other	0.2 (-25.3 to 25.7)	0.6 (-24.8 to 26.0)	0.96
Mean arterial pressure per 10 mm Hg	2.8 (0.7 to 4.9)	3.0 (0.9 to 5.1)	<0.01
History of hypertension	-0.0 (-9.1 to 9.0)	-0.3 (-9.7 to 9.1)	0.95
History of depression or schizophrenia	-1.2 (-12.7 to 10.2)	-3.3 (-14.6 to 8.0)	0.57

BDNF, brain-derived neurotrophic factor; CI, confidence interval.

BDNF and improved cognitive performance, compared with rats exposed to early (0-6 days) exercise.[65] In our study, low BDNF levels were associated with incomplete recovery at 6 months in individuals with TBI. Further studies are needed to validate this finding and to determine how well longitudinal BDNF values reflect recovery and clinical improvement post-TBI and the BDNF pathway as a therapeutic target.

Decreased circulating BDNF levels have been implicated in other non-TBI conditions including anxiety,[55] major depressive disorder,[56] schizophrenia,[57] and Alzheimer's disease.[66] However, these studies did not account for other potential confounders, such as age and gender. In our study, although control subjects with a history of a psychiatric disorder had lower median BDNF values than those without a history of a psychiatric disorder, this difference was not statistically significant. Additionally, after adjustment for age, gender, race, hypertension, diabetes and mean arterial pressure, history of psychiatric disorder was not an independent predictor of BDNF levels, whereas mean arterial pressure and gender were independent predictors of BDNF in control subjects. However, our findings regarding gender suggest that gender-specific cut-offs may be important in determining the reference values of BDNF. Since BDNF values increase during exercise, it is possible that in the case of sports-related concussions, increases in BDNF from exercise may mask a concussion-related decrease. Additional studies are needed to investigate BDNF levels in sports-related concussions.[67]

Limitations

Our study has a number of limitations. First, storage procedures for serum samples for JHH and SFGH TBI cases and JHH non-trauma controls were different. However, since our findings were reproduced in the TRACK-TBI Pilot cohort, it is unlikely that this discrepancy had an important influence on our study result. Further, BDNF increases with increased duration of storage at room temperature,[25] and that may explain why BDNF values in the JHH and SFGH cohorts are higher than BDNF in TRACK-TBI Pilot.

Additionally, the demographic distribution of our TBI cases was different from that of the non-TBI controls. However, the diagnostic accuracy of BDNF for discriminating between TBI cases and controls did not vary significantly after adjustment for potential confounders. Another major limitation is that the JHH controls had not been exposed to trauma. Since a common clinical challenge is to determine if TBI is present in patients who have been involved in automobile accidents, falls, or blast exposures, an important control group would be individuals exposed to orthopedic or systemic trauma but not head injury. Efforts to collect these ''other injury'' controls are under way.

In our validation cohort, the prevalence of traumatic intracranial abnormalities on head CT scan was much higher (47.2% of TRACK-TBI Pilot cases studied) than that reported in studies that are more representative of the population of ED patients evaluated for TBI.[68,69] Thus, examining the validity of our findings in cohorts that are more representative of ED patients evaluated for TBI will be important.

Conclusion

Serum BDNF discriminates between TBI cases and non-trauma controls with excellent diagnostic accuracy. Additionally, lower BDNF values are associated with incomplete recovery after TBI, and may be especially useful in identifying mild TBI patients who are likely to remain symptomatic at 6 months after injury.

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ORIGINAL ARTICLE

Association of a common genetic variant within ANKK1 with six-month cognitive performance after traumatic brain injury

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Abstract Genetic association analyses suggest that certain common single nucleotide polymorphisms (SNPs) may adversely impact recovery from traumatic brain injury (TBI). Delineating their causal relationship may aid in development
of novel interventions and in identifying patients likely to respond to targeted therapies. We examined the influence of the (C/T) SNP rs1800497 of ANKK1 on post-TBI outcome using data from two prospective multicenter studies: the

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in the Appendix in alphabetical order by last name

Registry: ClinicalTrials.gov Identifier NCT01565551

Electronic supplementary material The online version of this article
(doi:10.1007/s10048-015-0437-1) contains supplementary material,
which is available to authorized users.

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Citicoline Brain Injury Treatment (COBRIT) trial and Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot (TRACK-TBI Pilot). We included patients with ANKK1 genotyping results and cognitive outcomes at six months post-TBI ($n=492$: COBRIT $n=272$, TRACK-TBI Pilot $n=220$). Using the California Verbal Learning Test Second Edition (CVLT-II) Trial 1-5 Standard Score, we found a dose-dependent effect for the T allele, with T/T homozygotes scoring lowest on the CVLT-II Trial 1-5 Standard Score (T/T 45.1, C/T 51.1, C/C 52.1, ANOVA, $p=0.008$). Post hoc testing with multiple comparison-correction indicated that T/T patients performed significantly worse than C/T and C/C patients. Similar effects were observed in a test of non-verbal processing (Wechsler Adult Intelligence Scale, Processing Speed Index). Our findings extend those of previous studies reporting a negative relationship of the ANKK1 T allele with cognitive performance after TBI. In this study, we demonstrate the value of pooling shared clinical, biomarker, and outcome variables from two large datasets applying the NIH TBI Common Data Elements. The results have implications for future multicenter investigations to further elucidate the role of ANKK1 in post-TBI outcome.

Introduction

Traumatic brain injury (TBI) is a complicated injury in a complex organ. Each year in the USA, at least 2.5 million people suffer TBIs. This includes 52,000 deaths, 275,000 hospitalizations, and 1.365 million treated and released from an emergency department (ED) [1]. TBI is a contributing factor to 30 % of all injury-related deaths in the USA [1]. An estimated 3.2 to 5.3 million persons currently live with long-term physical, cognitive, and neuropsychiatric disabilities attributable to TBI [2]. Heterogeneity of the primary injury is complicated by a host of patient-specific factors that together determine clinical outcome [3]. Understanding how physiological factors influence patient outcome provides an avenue for identifying methods of clinical intervention, as well as the patients most likely to benefit. The advent of the Human Genome Project and genetic association analyses has allowed the identification of several polymorphic alleles of candidate genes that may signal disparate outcomes following TBI. However, examination of large numbers of genes results in high chance of type 1 error, underscoring the need for repeat studies of larger samples and high statistical power [4].

Cognitive deficits are among the leading sources of morbidity in TBI patients, and the underlying mechanisms are poorly understood. Patients presenting with similar injuries exhibit disparate patterns of cognitive impairment. The source of this variability is presently unknown but may involve genetic modulation as well as subtle morphometric differences in injury characteristics, highlighting the importance of investigating genetic differences that modulate cognitive function [5]. Previous studies have examined genes that modulate the dopaminergic pathway, which is critical to attention, memory, and executive function. As a result, the dopaminergic system is frequently targeted, through pharmacologic manipulation, to ameliorate chronic deficits in these areas following TBI [6].

Ankyrin repeat and kinase domain-containing 1 (ANKK1) is a candidate gene involved in dopamine transmission [7, 8]. In human adults, ANKK1 mRNA and protein is expressed in the central nervous system (CNS), exclusively in astrocytes [9]. The ANKK1 protein, also known as SgK288, shares structural homology with a family of serine/threonine receptor-interacting protein kinases (RIPKs) potentially responsible for signal transduction and cellular response modulation of dopaminergic reward processes [10].

A common single nucleotide polymorphism (SNP) in the ANKK1 gene may impact outcome after TBI [11, 12]. The C/T SNP rs1800497, also known as Taq1A, is located on chromosome band 11q23.1 in exon 8 of ANKK1 and causes a p. Glu713Lys amino acid change in the C-terminal ankyrin repeat domain, which is involved in protein-protein interaction [10]. Rs1800497 is located 10 kB downstream of the DRD2 gene. While unlikely to directly control DRD2 expression, it may be located within a regulatory region for a functional SNP in the DRD2 gene [10]. Positron emission tomography (PET) studies have shown that rs1800497 affects dopamine binding in the striatum in healthy volunteers [13]. Presence of a single T allele is associated with a 30–40 % reduction of dopamine D2 receptor (DRD2) density in the ventral striatum compared to homozygotes with C alleles, suggesting that T allele carriers may require increased dopaminergic tone to achieve similar levels of reinforcement and reward as C/C individuals. Studies have shown that one or two copies of the T allele of rs1800497 associates with disorders of reward deficiency such as alcohol dependence, smoking, and addictive behavior [14–17]. McAllister et al. found that rs1800497 allele status was associated with cognitive function following mild to moderate TBI ($N=141$: 93 TBI patients, 48 healthy controls) as defined by initial Glasgow Coma Scale (GCS) score of 9–15 and/or loss of consciousness (LOC) $\leq$24 h [11, 12]. The TBI group included 65 T-allele negative and 28 T-allele positive patients. T-allele positive patients showed worse episodic memory at 1 month post-TBI on the California Verbal Learning Test (CVLT) recognition trial, a result not observed in controls with the T allele. T-allele positive patients in the TBI group also exhibited slower performance on measures of response latency than those without the T allele [11, 12].

The present study extends this work in evaluating whether variation at rs1800497 within ANKK1 associates with verbal learning and non-verbal learning after acute TBI in a large multicenter cohort. We combined clinical and outcome data from two large prospective multicenter studies, The Citicoline Brain Injury Treatment Trial (COBRIT) [18, 19] and the Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot (TRACK-TBI Pilot) [20] to create the largest sample size to date of adult TBI patients with rs1800497 genotyping and six-month outcome testing after acute TBI ($N=492$). The merging of these two large datasets was made possible by their shared common standards—the National Institute of Neurological Disorders and Stroke (NINDS) TBI Common Data Elements (CDEs) [21]. We tested the primary hypothesis that the rs1800497 associates with reduced performance on the CVLT as previously described by McAllister et al. [11, 12] and assessed secondary endpoints and tertiary endpoints including a measure of non-verbal processing (Wechsler Adult Intelligence Scale Processing Speed Index (WAIS-PSI)).

Materials and methods

Study design

COBRIT is a multicenter, two-group, phase three, double-blind randomized placebo-controlled clinical trial conducted at eight U.S. Level I Trauma Centers [18, 19]. Inclusion criteria were patients with blunt force trauma to the head requiring inpatient hospitalization for TBI, with either: (1) Glasgow Coma Scale (GCS) score 3–12 and GCS motor score <6, or (2) GCS 3–12 with motor score 6 *or* GCS 13–15 *or* paralyzed after administration of paralytics as part of the clinical course with ≥1 of the following CT parameters: ≥10-mm diameter intraparenchymal hemorrhage, ≥5-mm extra-axial hematoma, subarachnoid hemorrhage visible on two or more 5-mm slices, or midline shift ≥5 mm. TRACK-TBI Pilot is a multicenter prospective observational study with patients recruited through convenience sampling at three U.S. Level I Trauma Centers [20]. Inclusion criteria were external force trauma to the head and clinically indicated head CT scan within 24 h of injury.

Exclusion criteria for both studies included positive pregnancy test result or known pregnancy, imminent death or current life-threatening disease, incarceration, or evidence of serious psychiatric and neurologic disorders that interfere with outcome assessment. Non-English speakers were not enrolled due to inability to participate in outcome assessments, which are normed and administered in English. The COBRIT study also excluded patients with bilaterally fixed and dilated pupils, those with prior TBI requiring hospitalization, concurrent enrollment in another study, and/or acetylcholinesterase inhibitor use within two weeks prior to injury. One trauma center (University of Pittsburgh) participated in both COBRIT and TRACK-TBI Pilot, but patients at this site were not co-enrolled into both studies.

The institutional review boards of all participating sites approved the protocols for each study. Patients were approached for informed consent before enrollment. For patients unable to give consent, due to their injury, consent was obtained from their legally authorized representative (LAR). Patients consented by LAR were approached for informed consent to continue participation while in the hospital or during follow-up assessment time-points.

These two studies enrolled a large number of TBI patients through acute and intermediate care to provide an ethnically and demographically diverse patient population. In TRACK-TBI Pilot, a comprehensive acute clinical profile was obtained from each patient in accordance with the National Institutes of Health (NIH) and NINDS CDEs across demographics, medical history, injury characteristics, acute hospital clinical care, and neuroimaging [22–26]. Enrollment in COBRIT began prior to the release of the NIH NINDS CDEs, but variables were collected in a standardized fashion with a high degree of concordance with the CDE effort [18], which enabled data pooling between the two studies. The pharmacological intervention in COBRIT consisted of daily enteral/oral citicoline (2000 mg) or placebo for 90 days. As the primary report by Zafonte et al. in 2012 found no association between citicoline use and improvement in functional and cognitive outcome [19], we did not pursue outcome analysis between treatment and control arms for this study.

Patient selection

All adult patients with complete 6-month outcomes and an acute blood biospecimen drawn for DNA were selected for this analysis from the COBRIT and TRACK-TBI Pilot studies. In both studies, patients without genotyping results and/or complete 6-month outcomes were excluded. Of 1213 total adult patients in COBRIT, 739 patients did not have blood genotyping results available and 202 of the remaining 474 patients had no or incomplete outcomes, leaving a final N of 272 patients for analysis. Of 650 total patients in TRACK-TBI Pilot, 51 patients were excluded from the non-acute TBI site and 27 patients were under the age of 18. Of the remaining 572 adult patients, 166 did not have blood genotyping results and 186 had genotyping but no or incomplete outcomes, leaving a final N of 220 for analysis. A comparison between included and excluded adult patients for this analysis, by study, is discussed in the Results section and in Online Resource 1 and 2. The distributions of demographic and clinical descriptors for COBRIT patients by treatment group are summarized in Online Resource 3.

Blood collection and genotyping

Specimen acquisition was performed as previously described [20]. In brief, blood samples for DNA genotyping analysis were collected via peripheral venipuncture or existing peripheral venous indwelling catheters within 24 h of injury. Samples were collected in BD Vacutainer K_2-EDTA vacutainer tubes, and subsequently aliquoted and frozen in cryotubes at −80 °C within 1 h of collection in accordance with recommendations from the NIH-CDE Biomarkers Working Group [25]. DNA was extracted from isolated leukocytes using the Wizard® Genomic DNA Purification Kit as described by the manufacturer (Promega, Madison, WI). The ANKK1 C/T SNP (rs1800497) was genotyped utilizing TaqMan®SNP Genotyping Assay as described by the manufacturer (Applied Biosystems, Carlsbad, CA). Patients were categorized by genotype: T/T, C/T, or C/C.

Outcome measures

The primary outcome measure was the California Verbal Learning Test, Second Edition (CVLT-II) Trials 1-5 Standard Score [27], which is one of the "core" TBI CDE outcome measures and was collected in both COBRIT and TRACK-TBI Pilot [28, 29]. The CVLT-II is a verbal learning and memory task in which there are five learning trials, an interference trial, an immediate recall trial, and a post-20 min recall trial. The CVLT-II Trials 1–5 Standard Score (CVLT-TSS) is normed for age and sex, and provides a global index of verbal learning ability [27]. The Wechsler Adult Intelligence Scale processing speed index (WAIS-PSI) was used as a secondary outcome measure [30]. Tertiary outcome measures collected across both studies include the Glasgow Outcome Scale-Extended (GOSE) [31], the Satisfaction with Life Scale (SWLS) [32], the Trail Making Test (TMT) Trail B minus Trail A Score (TMT B-A) [33], and the Brief Symptom Inventory 18 (BSI18) Global Severity Index Score (BSI18 GSI) [34].

Statistical analysis

Primary analysis assessed the impact of the T allele (T/T, C/T, C/C) on the chosen cognitive outcome measures. Group differences in demographic and clinical descriptors across ANKK1 genotypes (T/T, C/T, C/C) were assessed by Pearson's chi-squared test (X^2) for categorical variables and analysis of variance (ANOVA) for continuous variables. Row categories with average cell counts of less than 5 by ANKK1 were combined into a single row category during analysis. Fisher's exact test was used for comparisons with more than 20 % of individual cell counts less than 5. A two-way ANOVA was performed to assess the main effects of ANKK1 dose and study cohort (COBRIT vs. TRACK-TBI) as well as their interaction on 6-month CVLT-TSS. If the interaction was not significant, significant main effects were confirmed with a two-way ANOVA omitting the interaction term, using Tukey's post hoc test with multiple-comparison correction. Fisher's permutation test [35] was performed as a sensitivity analysis to address the unequal distribution of ANKK1 across races. Fifty thousand permutations, within study and race, were used to evaluate the effect of ANKK1. Significance was assessed at $\alpha=0.05$ for all analyses. Fisher's permutation test was performed using Statistical Analysis System (SAS), Version 9.4 (SAS Institute, Cary, NC). All other analyses were performed using Statistical Package for the Social Sciences (SPSS), Version 21 (IBM Corporation, Chicago, IL).

Results

Demographic and clinical descriptors

A total of 492 patients were included in the analysis (COBRIT $N=272$ (55 %), TRACK-TBI Pilot $N=220$ (45 %)). The overall mean age was 40 years old (standard deviation (SD) 16), and subjects were 75 % male (Table 1). The overall race distribution was 78 % Caucasian, 15 % African American/African, and 2 % or less of each of the other races. Mechanisms of injury were 35 % fall, 24 % motor vehicle accident, 16 % motorcycle/bicycle accident, 13 % assault, 7 % pedestrian struck by vehicle, 3 % struck by/against object, and 2 % other. TBI classification by emergency department (ED) arrival GCS was as follows: 21 % severe (GCS 3–8), 8 % moderate (GCS 9–12), and 71 % mild (GCS 13–15).

Comparison by study demonstrated that there was a lower proportion of African/American-African patients and higher proportions of non-Caucasian, non-African-American/African patients in TRACK-TBI Pilot (Caucasian 75 %, African-American/African 11 %, other 14 %) than in COBRIT (80, 19, and 1 %, respectively, $p<0.001$). Mechanism of injury differed by study ($p<0.001$) with more falls (43 vs. 28 %), fewer motor vehicle accidents (19 vs. 28 %), and fewer motorcycle/bicycle accidents (10 vs. 21 %) observed in TRACK-TBI Pilot than in COBRIT, respectively. COBRIT patients presented with lower GCS (28 % severe, 10 % moderate, 62 % mild) than TRACK-TBI Pilot patients (12 % severe, 5 % moderate, 83 % mild, $p<0.001$). No differences by study were observed in age, gender, or ANKK1 genotype (Table 2).

ANKK1 genotype distribution

ANKK1 genotype distribution was 8 % T/T ($N=40$), 36 % C/T ($N=175$), and 56 % C/C ($N=277$) consistent with the HapMap Phase III average across all races [36]. ANKK1 allelic frequencies ($T=0.26$, $C=0.74$) were found to be at or near Hardy–Weinberg equilibrium ($p=0.263$, Pearson X^2). T

Table 1 Demographic and clinical descriptors by ANKK1 genotype

Baseline variable	All patients	T/T	C/T	C/C	Sig. (*p*)
Age	*N*=492	*N*=40	*N*=175	*N*=277	0.861
Mean±SD	40±16	39±13	40±16	41±16	
Gender	*N*=492	*N*=40	*N*=175	*N*=277	0.404
Male	366	31 (9 %)	124 (34 %)	211 (58 %)	
Female	126	9 (7 %)	51 (41 %)	66 (52 %)	
Race[a]	*N*=489	*N*=40	*N*=174	*N*=275	<0.001
African-American/African	76	16 (21 %)	32 (42 %)	28 (37 %)	
American Indian/Alaskan	2	2 (100 %)	0 (0 %)	0 (0 %)	
Asian	11	2 (18 %)	5 (45 %)	4 (36 %)	
Caucasian	380	18 (5 %)	128 (34 %)	234 (62 %)	
Hawaiian/Pacific Islander	9	1 (11 %)	4 (44 %)	4 (44 %)	
More than one race	11	1 (9 %)	5 (45 %)	5 (45 %)	
Mechanism of Injury[a]	*N*=491	*N*=40	*N*=174	*N*=277	0.106
Motor vehicle accident	118	9 (8 %)	49 (42 %)	60 (51 %)	
Motorcycle/bicycle accident	79	5 (6 %)	33 (42 %)	41 (52 %)	
Pedestrian struck by vehicle	33	2 (6 %)	14 (42 %)	17 (52 %)	
Fall	170	11 (6 %)	50 (29 %)	109 (65 %)	
Assault	66	10 (15 %)	17 (26 %)	39 (59 %)	
Struck by/against object	14	1 (7 %)	5 (36 %)	8 (57 %)	
Other	11	2 (18 %)	6 (55 %)	3 (27 %)	
ED arrival GCS	*N*=489	*N*=40	*N*=175	*N*=274	0.097
Mild (13–15)	348	34 (10 %)	125 (36 %)	189 (54 %)	
Moderate (9–12)	38	2 (5 %)	18 (47 %)	18 (47 %)	
Severe (3–8)	103	4 (4 %)	32 (31 %)	67 (65 %)	

Distribution of demographic and clinical descriptors by ANKK1 genotype. Row percentages are shown for categorical variables (may not equal exactly 100 % due to independent rounding). Statistical significance (*p*) is assessed using the Pearson chi-squared statistic or Fisher's exact test for categorical variables and ANOVA for continuous variables, by ANKK1 genotype with α=0.05. *ED* Emergency Department, *GCS* Glasgow Coma Scale

[a] Row categories with average cell counts of less than 5 are combined into a single row category during analysis

allele distribution differed across races (*p*<0.001) but conformed to known HapMap Phase III frequencies [36]. Distributions across the two primary race groups in this study were assessed: Caucasians (5 % T/T, 34 % C/T, 61 % C/C) did not differ from the expected CEU HapMap (5 % T/T, 28 % C/T, 66 % C/C (*p*=0.291)), and African American/Africans (21 % T/T, 42 % C/T, 37 % C/C) did not differ from the expected YRI HapMap (16 % T/T, 50 % C/T, 34 % C/C (*p*= 0.606)). HapMap comparisons for ANKK1 were not performed for the other races due to small sample sizes of *n*≤ 10. No differences in ANKK1 genotype distribution were observed by age, gender, mechanism of injury, or GCS.

Comparison of descriptors between included and excluded patients by study

In both studies, there was a higher proportion of African-American/African patients included in this analysis (COBRIT *N*=270, 80 % Caucasian, 19 % African-American/African, 1 % other; TRACK-TBI Pilot *N*=220, 75 % Caucasian, 11 % African-American/African, 14 % other) compared to patients not included (COBRIT *N*=938, 83 % Caucasian, 13 % African-American/African, 4 % other, *p*=0.033; TRACK-TBI *N*=348, 85 % Caucasian, 6 % African-American, 9 % other, *p*=0.043). The included COBRIT patients had less severe injuries by GCS (*N*=271, 28 % severe, 11 % moderate, 62 % mild) compared to those not included (*N*=936, 39 % severe, 10 % moderate, 51 % mild, *p*=0.004). The included TRACK-TBI Pilot patients were younger (*N*=220, mean 41, SD 16) compared to those not included (*N*=352, mean 46, SD 19). No differences in other baseline descriptors or ANKK1 genotype distribution were observed between included and excluded adult patients within each study (Online Resource 1 and 2).

Comparison of descriptors between COBRIT treatment and control arms

The COBRIT patients included in this analysis (*N*=272) distributed evenly across citicoline (*N*=137 (50 %)) and placebo arms (*N*=135 (50 %)). No differences in any demographic and clinical descriptors were observed by treatment arm (Online Resource 3).

Table 2 Demographic and clinical descriptors by study

Baseline variable	COBRIT	TRACK-TBI Pilot	Sig. (p)
Age	N=272	N=220	0.453
Mean±SD	40±15	41±16	
Gender	N=272	N=220	0.072
Male	211 (78 %)	155 (70 %)	
Female	61 (22 %)	65 (30 %)	
Race[a]	N=270	N=219	<0.001
African-American/African	51 (19 %)	25 (11 %)	
American Indian/Alaskan	0 (0 %)	2 (1 %)	
Asian	2 (1 %)	9 (4 %)	
Caucasian	216 (80 %)	164 (75 %)	
Hawaiian/Pacific Islander	0 (0 %)	9 (4 %)	
More than one race	1 (0 %)	10 (5 %)	
Mechanism of injury	N=272	N=219	<0.001
Motor vehicle accident	77 (28 %)	41 (19 %)	
Motorcycle/bicycle accident	58 (21 %)	21 (10 %)	
Pedestrian struck by vehicle	13 (5 %)	20 (9 %)	
Fall	76 (28 %)	94 (43 %)	
Assault	33 (12 %)	33 (15 %)	
Struck by/against object	8 (3 %)	6 (3 %)	
Other	7 (3 %)	4 (2 %)	
ED arrival GCS	N=271	N=218	<0.001
Mild (13–15)	168 (62 %)	180 (83 %)	
Moderate (9–12)	27 (10 %)	11 (5 %)	
Severe (3–8)	76 (28 %)	27 (12 %)	
ANKK1 genotype	N=272	N=220	0.193
T/T	17 (6 %)	23 (11 %)	
C/T	102 (38 %)	73 (33 %)	
C/C	153 (56 %)	124 (56 %)	

Distribution of demographic and clinical descriptors by study. Column percentages are shown for categorical variables (may not equal exactly 100 % due to independent rounding). Statistical significance (p) is assessed using the Pearson chi-squared statistic or Fisher's Exact Test for categorical variables, and ANOVA for continuous variables, by ANKK1 genotype with α=0.05. *ED* Emergency Department, *GCS* Glasgow Coma Scale

[a] Row categories with average cell counts of less than 5 are combined into a single row category during analysis

Relationship of ANKK1 to CVLT-TSS

Our analyses were designed to address potential confounding created by pooling COBRIT and TRACK-TBI Pilot data for the effects of the following: (1) the particular study and (2) interaction between ANKK1 and the particular study on CVLT-TSS. First, we performed a two-way ANOVA with CVLT-TSS as the dependent variable to assess the main effects of ANKK1 and study, plus the interaction term ANKK1 X study. Table 3 shows that ANKK1 had a statistically significant association at α=0.05 with CVLT-TSS ($F(2, 486)$=4.964, p=0.007), while particular study and ANKK1 X

Table 3 Association of ANKK1 genotype and study with 6-month CVLT-TSS

Source	Type III sum of squares	df	Mean square	F	p value
Corrected model	1773.6	5	354.7	1.993	0.078
ANKK1	1767.4	2	883.7	4.964	0.007
Study	54.2	1	54.2	0.304	0.581
ANKK1 X study	35.0	2	17.5	0.098	0.906
Error	86517.8	486	178.0		

Two-way ANOVA to assess the main effects of ANKK1 genotype (abbreviated as ANKK1) and Study (COBRIT or TRACK-TBI Pilot) plus the interaction term (ANKK1 X Study) on six-month CVLT-TSS as the dependent variable in the model. Significant assessed at α = 0.05

CVLT-TSS California Verbal Learning Test, Second Edition Trials 1-5 Standard Score, *df* degrees of freedom

study did not. We then re-ran the model, omitting the interaction term, to confirm the significant association between ANKK1 and CVLT-TSS ($F(2,486)$=4.893, p=0.008), and not between particular study and CVLT-TSS ($F(1,486)$= 0.117, p=0.732). We performed Tukey's post-hoc test for ANKK1 in the same model to assess for differences in CVLT-TSS across the three ANKK1 genotypes. Figure 1
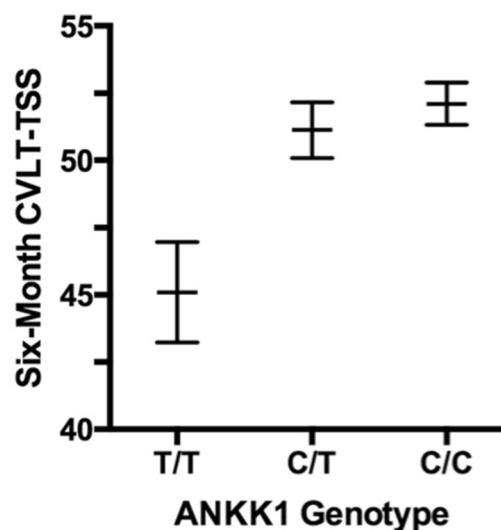
ANKK1 Genotype (I vs. J)	Mean CVLT-TSS (SE) (I vs. J)	MD (95% CI) (I minus J)	p-value
T/T vs. C/T	45.1 (1.9) vs. 51.1 (1.0)	-6.0 (-11.5, -0.5)	0.027
T/T vs. C/C	45.1 (1.9) vs. 52.1 (0.8)	-7.0 (-12.3, -1.7)	0.006

Fig. 1 Comparison of 6-month CVLT-TSS means across ANKK1 genotypes. Graph shows 6-month CVLT-TSS mean±SE by ANKK1 genotype. Tukey's post hoc test was used to assess mean differences (MD) in CVLT-TSS between genotypes. Only significant MDs at α=0.05 are shown in the table. Mean difference is calculated by the mean CVLT-TSS of the first genotype (I) minus that of the second genotype (J). *CVLT-TSS* California Verbal Learning Test, Second Edition Trials 1–5 Standard Score, *SE* standard error, *CI* confidence interval

shows the CVLT-TSS means by ANKK1, and that mean CVLT-TSS of T/T patients differed significantly from that of C/T and C/C patients, with a mean decrease of 6.0 points against C/T and 7.0 points against C/C.

Based on our initial descriptive statistics (Table 1), there were subpopulation differences in the distribution of ANKK1 genotypes across races. As a sensitivity analysis, we ran Fisher's permutation test as a distribution-free alternative to the parametric model [35]. The association between ANKK1 and six-month CVLT-TSS remained significant ($p=0.026$) when controlling for race and particular study.

Exploratory analysis of ANKK1 on other outcome measures

To explore the common six-month outcome measures in our pooled multicenter dataset, we assessed the association between ANKK1 genotype on a non-verbal cognitive test, the WAIS-PSI, as well as with four other measures: GOSE, SWLS, TMT B-A, BSI18 GSI. We performed identical analyses as above to assess the main effect of ANKK1 genotype and particular study, plus the interaction factor ANKK1 X study, using two-way ANOVA with each outcome measure as the dependent variable. There was a significant association at $\alpha=$ 0.05 between ANKK1 and WAIS-PSI ($F(2,486)=3.225$, $p=$ 0.041), and particular study and WAIS-PSI ($F(1,486)=7.01$, $p=0.008$), with no effect of ANKK1 X study. No significant pairwise differences at $\alpha=0.05$ were observed in WAIS-PSI means across ANKK1 (T/T: 94.1, SE 2.5; C/T: 95.9, SE 1.3; C/C: 98.8; SE 0.9) on Tukey's post-hoc test. Mean WAIS-PSI scores in COBRIT were lower than in TRACK-TBI Pilot (COBRIT: 95.9, SE 1.0; TRACK-TBI Pilot 99.3, SE 1.1, $p=$ 0.02). There was no significant association between ANKK1, study or ANKK1 X study with GOSE, SWLS, or TMT B-A. There was a marginal association at $\alpha=0.05$ between ANKK1 and BSI18 GSI ($F(2,486)=3.0$, $p=0.052$), with no effect of particular study or ANKK1 X study. On Tukey's post hoc test, BSI18 GSI means (T/T 60.1, SE 2.1; C/T 54.7, SE 0.9; C/C 56.3, SE 0.7) differed significantly at $\alpha=0.05$ between T/T and C/T only (95 % CI 0.3 to 10.4, $p=0.036$).

Discussion

Over the past decade, genetic association studies have contributed to our understanding of the molecular mechanisms of multiple common human diseases, including Alzheimer disease, heart disease, and diabetes, among others [37–42]. In each case, molecular mechanisms suspected to be involved in disease pathogenesis based on preclinical or pathologic studies were confirmed by human genetics. In addition, human genetic association studies have uncovered new molecular pathways previously unsuspected to play a role in disease

pathogenesis [43–47]. The overwhelming majority of these genetic discoveries, however, have applied to disease risk [48–52]. TBI presents special challenges for genetic association studies [53]. First, there is a prominent and stochastic environmental factor: the traumatic injury. Second, premorbid personality and developmental factors play a clear role in recovery from injury. Thus, in order to identify molecular pathways in resilience to or recovery from TBI, large sample sizes and collection of comprehensive data, which allow for consideration of premorbid factors and assessment of injury severity, are essential [54, 55]. The use of CDEs is fundamental to the success of these efforts and the NIH-NINDS TBI CDEs were designed to address this need [21]. Investigators of COBRIT and TRACK-TBI Pilot were among the leaders in this effort, and the present study was feasible because of the high degree of overlap between the assessment tools and outcome measures utilized in the two studies.

Our robust sample permitted confirmation of the hypothesis concerning the effects of the T allele on cognitive outcome. Indeed, we found an association between ANKK1 and poorer performance on 6-month CVLT-TSS specifically tied to the T/T genotype. The C/T group alone did not show any differences from the C/C group on CVLT-TSS. Although this does not align perfectly with previous findings in TBI, where T-allele carriers showed worse performance on an episodic memory task of the CVLT, our overall result remains more confirmatory than divergent. McAllister et al. reported only one T/T individual in a sample size of 141, which could not enable a T-dose-dependent analysis. The distribution ANKK1 genotypes in our analysis approaches that of the general population according to HapMap Phase III and therefore allows us more statistical power to investigate the differential relationships between genotype and cognition. Secondly, it may be that differential genotypic associations with specific symptoms are more easily identified on specialized verbal memory trials such as the CVLT recognition task while the deleterious effect of a double dose of T allele manifest on the CVLT-TSS, a more highly generalizable and normative global index of verbal learning ability.

Our study reinforces the benefits of pooling multicenter trials into a unified data commons. There were no differential study effects by COBRIT and TRACK-TBI Pilot, nor were there ANKK1 X study interactions, on six-month verbal learning. This validates data sharing as a mechanism to raise statistical power for hypothesis testing and increases our confidence in the associations of ANKK1 T/T with verbal learning across a large, heterogeneous TBI population.

As well, merging COBRIT and TRACK-TBI Pilot data effectively captures patients across the entire TBI spectrum. As COBRIT excluded patients with GCS 13–15 presenting with negative head CTs, it targeted patients with more moderate and severe TBI whereas TRACK-TBI Pilot enrolled patients with similar TBI incidence as reported in literature and the

population, which is predominantly mild [56, 57]. Indeed, COBRIT patients in the current analysis presented with more severe TBI compared to TRACK-TBI Pilot, and this difference may account for the observed differences by study in some of our analyses of secondary outcomes. For example, the study effect on WAIS-PSI scores reached significance. It is also interesting to note the marginal signal of ANKK1 T/T with the BSI18 GSI, which corroborates the range of studies interrogating ANKK1 in the context of neuropsychiatric disorders.

Our study has clarified several key areas identified by McAllister et al. as areas of further investigation concerning the relationship of ANKK1 with TBI outcome [11, 12]. The authors questioned whether their results would hold in a larger, more diverse racial and ethnic population, with varying injury severity and in outcomes at a longer post-injury interval. By utilizing two multicenter studies (COBRIT: eight centers, TRACK-Pilot: three centers, one center participated in both studies), the sample size was expanded to encompass a total of 10 Level I trauma centers across the USA. This heterogeneous population covers the full severity spectrum from concussion to coma, which previous studies did not have an opportunity to evaluate in the context of ANKK1. Regarding outcomes, McAllister et al. were only able to access CVLT at 1-month post-injury and expressed concern about generalizability at later timepoints. With a larger multicenter sample and long-term follow-up (the 6-month clinical standard), the present study is more resilient to local demographic and practice effects providing a strong replication test of McAllister et al.'s results.

Limitations

Although we have improved upon the breadth and generalizability of previous studies, we recognize several limitations in the current analysis. First, we could not fully account for the impact of TBI pathology and lesion types on recovery, the lack of pre-injury psychometric tests, other genetic predispositions, and non-TBI control groups. As our primary analysis was confirmatory in nature, we pursued similar inclusion criteria as McAllister et al. for general TBI and did not explore the structure-function implications of ANKK1 with intracranial lesion types or baseline mental health variables. Given the heterogeneity of TBI, subjects may never be perfectly matched by type, location, and extent of injury. Despite this fact, convincing evidence of genetic association can be clarified by sufficiently large sample sizes. The ability to comment on causative or confounding relationships between ANKK1 and pre- or post-injury risk factors is beyond the scope of the current analysis. As T/T has been associated with propensity for addiction and poor coping strategies [8, 14–17, 58, 59], the acquisition and analysis of detailed pre-injury addictive behavior, post-acute treatment, and recovery variables are relevant next steps in delineating the contribution of ANKK1 to both TBI risk and outcome variability. We are also constrained by

the lack of genome-wide data, which makes it difficult to fully control for population stratification, as evidenced by the observed differences for patients who met the inclusion criteria for this analysis compared their excluded counterparts in COBRIT and TRACK-TBI Pilot. The proportion of T/T within our sample is still rather small, limiting our ability to assess whether there is a differential influence of ANKK1 genotypes on other domains of outcome, or in different races. The robustness of the association between ANKK1 and a given outcome domain such as working memory or processing speed, which encompasses multiple individual outcome measures, can be interrogated using multivariate integration and correlated with specific injuries in the dorsolateral prefrontal cortex—where working memory processes are known to be confined [60–62]. Work of this type is ongoing in the TRACK-TBI consortium.

In analyzing patients with full outcomes, there is an inherent risk of selecting for patients able to return for follow-up. For example, in our study, the COBRIT patients with genotyping and complete six-month outcomes presented with less severe injuries than those who had incomplete outcomes. This may be attributable in part to better cognition and functional ability to return for follow-up. As observed in TRACK-TBI Pilot, patients of younger age may be more mobile and/or available to return for full outcomes assessment. In some ways, the selection bias relates to the primary goal of this analysis, which was to assess the association of ANKK1 with outcome measures common to both studies and hence contingent on patients with valid scores. It is difficult to capture reasons for incomplete outcomes in patients who are lost to follow-up, as in many cases contact is never made.

The molecular mechanism and active location of ANKK1 remains a topic of ongoing study, with further experiments needed in cellular and animal models, as well as human trials. There is a need to examine gene-gene interaction with other loci of susceptibility for prognostic phenotyping within the dopaminergic system to elucidate an ANKK1 molecular pathway in local CNS physiology, contingent on detailed structure-function analysis from the comprehensive mapping of the human connectome [63]. Alternatives to the limitations of conventional imaging modalities such as CT are being explored with TRACK-TBI Pilot data. Early results indicate that prediction models including contusion on 3T MRI and axonal injury by diffusion tensor imaging (DTI) surpass other predictors for global outcome prediction in a subset of patients after mild TBI [64]. Advanced diffusion imaging modalities targeting the dorsal prefrontal cortex have been reported for healthy and diseased states [65–69]. Increased precision in characterizing regional pathophysiology will enable more objective control of injury type and severity in order to distill the specific mechanism by which ANKK1 modulates working memory, as a subset of the disparate patterns of cognitive impairment observed in the current TBI classification system of mild, moderate, and severe. In a broader sense, further

development of classification approaches based on quantitative morphometry [70], in conjunction with appropriate computational methods [71] and data integration processes [72], will aid in deconstructing the contribution of genetic modulation to multidimensional domains of outcome after TBI.

Greater sample size and more extensive genotyping will overcome our current limitations to allow for stratification across known genetic profiles and TBI severities, as well as raise statistical power to levels appropriate for phase III clinical trials. We successfully pooled COBRIT and TRACK-TBI Pilot data through outcome measures common to both studies, but we were still constrained in our scope of data pooling. Clearer evaluations of the effects of risk factors and predictors of TBI outcome, including ANKK1 and other SNPs, await the expanded initiatives of current multicenter studies such as the Transforming Research and Clinical Knowledge in TBI study (TRACK-TBI) [73] and the Collaborative European NeuroTrauma Effectiveness Research in TBI study (CENTER-TBI) [74], which will enroll 3000 and 5000 patients with controls, respectively, over the next five years, using the expanded Version 2 of the NIH-NINDS TBI CDEs [21, 75]. Adopting an international approach [76] to this standardized set of variables with wide scope, utility, and applicability will allow us to converge and leverage research efforts to achieve the sample sizes we truly need for delineating the effects of the ANKK1 polymorphism in TBI.

Conclusions

In the largest prospective multicenter study to date examining the incidence of the rs1800497 SNP in TBI, enabled by data pooling of shared common variables, we report that the ANKK1 T/T genotype associates with poorer verbal learning performance on CVLT-TSS at six months post-injury across the spectrum of TBI severity. With the augmented statistical power of this analysis, successful replication of the association between ANKK1 and cognition reinforces the potential implication of a DRD2-dependent biological mechanism underlying cognitive performance after TBI.

Compliance with Ethical Standards

Appendix

COBRIT investigators

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Plasma Anti-Glial Fibrillary Acidic Protein (GFAP) Autoantibody Levels During the Acute and Chronic Phases of Traumatic Brain Injury - A TRACK-TBI Pilot Study

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Running Head: Anti-GFAP Autoantibody in Plasma in TBI Subjects

Manuscript Category: Short Communications

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ABSTRACT

We recently described a subacute serum autoantibody response towards glial fibrillary acidic protein (GFAP) and its breakdown products 5 to 10 days after severe traumatic brain injury (TBI). Here, we expanded our anti-GFAP autoantibody (AutoAb[GFAP]) investigation to the multicenter observational study Transforming Research and Clinical Knowledge in TBI Pilot (TRACK-TBI Pilot) to cover the full spectrum of TBI (GCS 3-15) by using acute (<24 h) plasma samples from 196 acute TBI patients admitted to 3 Level I trauma centers, and a second cohort of 21 chronic TBI subjects admitted to inpatient TBI rehabilitation. We find that acute subjects self-reporting prior TBI with loss of consciousness (LOC) (n=43) had higher day 1 AutoAb[GFAP] (mean±SE: 9.11±1.42; n=43) than normal controls (2.90±0.92; n=16; p=0.032) and acute subjects reporting no prior TBI (2.97±0.37; n=106; p<0.001), but not acute patients reporting prior TBI without LOC (8.01±1.80; n=47; p=0.906). These data suggest that while exposure to TBI may trigger the AutoAb[GFAP] response, circulating antibodies are elevated specifically in acute TBI patients with prior history of TBI. AutoAb[GFAP] levels for chronic TBI patients (average post-TBI time 176 days or 6.21 months) were also significantly higher (15.08±2.82; n=21) than normal controls (p<0.001). These data suggest a persistent upregulation of the autoimmune response to specific brain antigen(s) in the subacute to chronic phase following TBI, as well as following repeated TBI insults. Hence AutoAb[GFAP] may be a sensitive assay to study the dynamic interactions between post-injury brain and patient-specific autoimmune responses across acute and chronic settings after TBI.

Key words: autoimmunity, biomarkers, traumatic brain injury, autoantibody, glia

INTRODUCTION

Traumatic brain injury (TBI) causes transient opening of brain-blood barrier which is often followed by neural cell damage or death. During the acute phase of TBI, a number of brain-specific proteins are released into the cerebrospinal fluid and/or blood (serum/plasma). A partial list includes neuronal proteins [ubiquitin-C-terminal hydrolase-L1 (UCH-L1)], microtubule associated protein tau (MAPT/Tau), neuron specific enolase (NSE), axonal proteins [neurofilament-H, αII-spectrin breakdown products (SBDPs)], dendritic protein [MAP2], glial proteins [glial fibrillary acidic protein (GFAP), S100β] oligodendrocyte proteins [myelin basic protein (MBP)], and endothelial cell derived proteins [e.g. von Willebrand Factor (VWF)].[1-4] As the brain is a site of immune-privilege, most of these proteins are not generally accessible to the immune system. TBI represents a situation where high concentrations of brain proteins are transiently released into the circulation and become accessible to the immune system.

Previous reports have documented brain-directed autoimmunity in neurological and neurodegenerative diseases such as Alzheimer's disease, stroke, epilepsy, spinal cord injury and paraneoplastic syndromes.[5-11] In human TBI, however, autoimmunity has only been examined in a limited way and focused on autoantibodies against preselected antigens such as MBP, S100B, and glutamate receptors.[12-18] Tanriverdi et al. showed the presence of anti-pituitary antibodies in patient serum 3 years after head trauma.[19-23] Recently Marchi et al. demonstrated that anti-glial protein S100b autoantibody levels are elevated in football players with repeated concussions.[24,25] In parallel, we recently reported a rather unexpected immunodominant autoantibody response to GFAP and its breakdown products (BDPs) in a subset of severe TBI subjects.[26]

Based on our previous anti-GFAP-autoantibody study,[26] we observed that GFAP appeared to be a dominant brain-derived autoantigen following severe TBI. We hypothesized that TBI

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causes protease-mediated GFAP-breakdown product (GFAP-BDP) formation in injured glial cells. This is followed by the subsequent release of GFAP-BDPs in substantive quantity through a compromised brain-blood barrier into the circulation.[24,27-29] This combination allows GFAP and GFAP-BDPs to become accessible to and recognized by the immune cells as non-self-proteins, triggering autoantibody response in those individuals. While GFAP is an intracellular antigen and the central nervous system (CNS) is normally considered immune-privileged, it is still conceivable that autoantibodies can gain assess to the CNS tissue where such an antigen is localized. For example MBP, myelin oligodendrocyte glycoprotein (MOG) and other intracellular myelin proteins in the spinal cord appear to be attacked by immune system in multiple sclerosis and other demyelination diseases.[30] Hence it is possible that an autoantibody specifically targeting a major brain protein such as GFAP might trigger a persistent autoimmune activation, which could negatively impact on long-term recovery from TBI. Thus we sought to expand our anti-GFAP autoantibody (AutoAb[GFAP]) investigation to the Transforming Research and Clinical Knowledge in TBI Pilot (TRACK-TBI Pilot) study,[31] a multicenter observational study that covers the full spectrum of TBI (GCS 3-15) with acute (<24 h) plasma samples available from 196 acute TBI patients admitted to Level I trauma centers, as well as a second cohort of 21 chronic TBI subjects admitted to an inpatient rehabilitation center.

METHODS

TBI Subjects: Subjects with acute traumatic brain injury were identified and recruited upon arrival at one of three Level I trauma centers and one inpatient TBI rehabilitation center as part of the multicenter prospective TRACK-TBI Pilot study.[31] Study protocols were approved by the institutional review boards of participating centers (acute sites: San Francisco General Hospital (SFGH); University of Pittsburgh Medical Center (UPMC), University Medical Center Brackenridge (UMCB); rehabilitation site: Mount Sinai Rehabilitation Center (MSRC)). All participants or their legal authorized representatives provided written informed consent. At follow-up outcome time points, participants previously consented by legally authorized representative were consented for continuation in the study if neurologically improved to be capable of self-consent.

To be eligible for the TRACK-TBI Pilot study, acute TBI patients presented within 24 h of injury to the emergency department and have history of trauma to the head sufficient to triage to non-contrast head computed tomography (CT) scan using the American College of Emergency Physicians/Centers for Disease Control (ACEP/CDC) evidence-based joint practice guideline, while chronic TBI patients had sufficient neurologic impairment to triage to inpatient TBI rehabilitation. Details of loss of consciousness (LOC), amnesia and source of trauma were recorded upon screening, and informed consent was obtained. Glasgow Coma Scale (GCS) score was assessed by a neurosurgeon at admission and was reconfirmed by study personnel at the time of biomarker collection. For chronic TBI, plasma samples were collected upon presentation to rehabilitation at MSMC with an average post-injury time of 188 days (6.2 months). We further identified acute TBI subjects with self-reported prior TBI with or without LOC (see **Table 1**).

Biosample Collection: Blood samples from acute TBI were collected from subjects who

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consented to genetic and proteomic analysis within 24 h of injury (n=196). Blood samples from chronic TBI were collected at the indicated time point. Plasma was extracted as supernatant after centrifugation of whole blood in EDTA blood tubes for 5-7 min at 4,000 RPM according to the National Institutes of Health / National Institute of Neurological Disorders and Stroke (NIH/NINDS) TBI Common Data Elements Biospecimens and Biomarkers Working Group recommendations.[32] In addition, 16 commercial control plasma samples collected with EDTA blood tubes (Bioreclamation Inc., mean ± standard deviation (SD) 39.1 ± 17.2 years old) were age-matched with the acute (n=196; 42.1 ± 18.1 years old) and chronic TBI (n=21; age 44.4 ± 20.5 years old) samples and assayed for AutoAb[GFAP].

Measurement of AutoAb[GFAP]: To detect and quantify AutoAb[GFAP] levels in biosamples, we used our previously published manifold autoantibody immunoblotting assay format[26] (see **Supplementary Figure 1** for assay set-up). Briefly, human brain GFAP protein or human brain fraction enriched in GFAP protein (20 ug) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-20% Tris-glycine 1-well gel and electro-transferred to polyvinylidene fluoride (PVDF) membrane. PVDF membranes were then clamped into the Mini-Protean II Multiscreen apparatus (Bio-Rad), and individual lanes were blocked and probed with human sera diluted at 1:100, unless otherwise noted. This manifold autoantibody immunoblot assay[26] requires only a 1/100 dilution (e.g. 1 uL in 100 uL). We serially diluted the plasma to verify that the signal is plasma concentration-dependent provided that it is within the optical density (OD) readings for the spectrometer. Secondary antibodies used were either alkaline phosphatase (AP)-conjugated goat anti-human immunoglobulin G (IgG) or AP-conjugated donkey anti-human IgG diluted 1:10,000 (Jackson ImmunoResearch). Blots were developed at room temperature with substrate 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride (BCIP-NBT) solution for 10 min. We also routinely performed in-solution preabsorption with GFAP protein (2 ug / 100 uL) as a control study. The

bands of interest on the blotting membrane disappear after preabsoprtion (data not shown). Quantification of autoantibody reactivity on immunoblots was performed via computer-assisted densitometric scanning (Epson 8836XL high-resolution scanner and NIH Image J densitometry software). Autoantibody levels were expressed in arbitrary densitometry units. Values are reported as mean and standard error (SE) unless stated otherwise. Analysis of variance (ANOVA) was used for multi-group analysis; Tukey's post-hoc test used to assess mean differences between subgroups as well as distinguish homogeneous subsets. Statistical significance was assessed at p < 0.05. Statistics were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and Statistical Analysis System (SAS) version 9.2, (SAS Institute, Inc., Cary, NC, USA) unless stated otherwise.

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RESULTS

Anti-GFAP autoantibody levels in acute plasma samples from TRACK-TBI Pilot study.

As we previously identified a dominant autoantibody response to glial intermediate filament protein GFAP among severe TBI patients,[26,33-35] here we sought to expand these findings by utilizing the TRACK-TBI Pilot study cohorts and plasma samples.[1,36,37] Of 586 acute TBI subjects from TRACK-TBI Pilot, we identified 196 subjects with available acute plasma samples (collected within 24 h of injury) for this autoantibody study. Study patients covered the range of initial GCS of 3-15, which are reported with age, gender, and admission head CT distributions in **Table 1**. The TRACK-TBI Pilot study also recorded self-reported prior TBI history (apart from the index TBI of enrollment), with the following categories: no prior TBI (n=106), prior TBI without LOC (n=47), and prior TBI with LOC (n=43) (**Table 1**).

Autoantibodies reacting with intact GFAP (50 kDa) and its various breakdown products (48-38 kDa) were assayed using quantitative manifold immunblotting developed previously.[3,26] The distribution for AutoAb[GFAP] (mean ± SE) was 2.90 ± 0.92 units for healthy controls, 2.97 ± 0.37 units for acute TBI patients reporting no prior TBI, 8.01 ± 1.80 units for acute TBI patients reporting prior TBI without LOC, and 9.11 ± 1.42 units for acute TBI patients reporting prior TBI with LOC. ANOVA showed a significant difference across groups ($p < 0.001$); Tukey's post-hoc test demonstrated that healthy controls and acute TBI patients reporting no prior TBI constituted a statistically different subgroup in AutoAb[GFAP] levels than acute TBI patients reporting prior TBI either with or without LOC (**Figure 1**). Specifically, acute TBI patients reporting prior TBI with LOC showed significantly elevated AutoAb[GFAP] levels than healthy controls (mean increase 6.21 ± 2.26, $p = 0.032$) and patients reporting no prior TBI (mean increase 6.14 ± $p < 0.001$), but not with acute TBI patients reporting prior TBI without LOC (mean increase 1.10 ± 1.62, $p = 0.906$); acute TBI patients reporting prior TBI without LOC showed significantly elevated AutoAb[GFAP] levels when compared with acute TBI patients reporting no prior TBI

(mean increase 5.04 ± 1.35, p = 0.001), but not with healthy controls (mean increase 5.11 ± 2.23, p = 0.103). No difference was observed in AutoAb[GFAP] between healthy controls and acute TBI patients reporting no prior TBI (mean increase 0.07 ± 2.07, p = 0.999).

In previous reports we have assayed the same patient plasma samples for GFAP (and its BDP) levels.[36,37] Thus, we examined whether there is a correlation between GFAP antigen levels and AutoAb[GFAP] levels in these samples. As expected, we did not find a correlation between the two (*data not shown*).

In addition, we sought to examine acute AutoAb[GFAP] distributions across different initial GCS scores. Due to relatively small number of samples for those with lower GCS, by convention we grouped acute patients to three GCS categories for autoantibody comparison purposes: GCS 3-8 (N=12; mean ± SE: 3.35 ± 0.87), GCS 9-12 (N=6, 4.37 ± 1.59), GCS 13-15 (n=169, 6.16 ± 0.72). Results on ANOVA showed no statistically significant differences in acute AutoAb[GFAP] levels across the three GCS categories (p = 0.197).

We also examined acute AutoAb[GFAP] distributions by presence of intracranial pathology on admission head CT, across categories of "no intracranial pathology" (N=108), "extraaxial pathology only" (n=22), "intraaxial pathology only" (n=24), and "both extraaxial and intraaxial pathology" (N=42). Results on ANOVA showed no statistically significant differences in acute AutoAb[GFAP] levels across the four categories (mean ± SE: 6.48 ± 0.87; 5.02 ± 2.01; 5.72 ± 2.04; 3.22 ± 0.49 respectively; p = 0.197). To further explore the relationship between pathological injury severity and AutoAb[GFAP] , we analyzed the distribution of AutoAb[GFAP] across Marshall CT categories. Due to small numbers of individual Marshall CT scores of 3 (N=9), 4 (N=2), 5 (N=12), and 6 (N=1), we combined Marshall Score 3-6 into a single category "3+". AutoAb[GFAP] distributions were as follows: Marshall 1 (N=96, mean ± SE: 6.20

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± 0.91), Marshall 2 (N=78, 5.57 ± 0.96), Marshall 3+ (N=22, 2.47 ± 0.64) and showed no statistically significant differences across Marshall CT categories (p = 0.148).

Anti-GFAP autoantibody levels in chronic plasma samples from TRACK-TBI Pilot study.

We previously demonstrated that post-TBI serum AutoAb[GFAP] shows a delayed increase, beginning about 5-6 days after severe TBI and sustained to at least 10 days.[26,38] Here, we examined AutoAb[GFAP] levels in chronic TBI plasma samples collected from 21 subjects during rehabilitation. The demographics of these subjects are tabulated in **Table 1**. Initial GCS and CT Marshall scores or GOS-E data were unavailable for chronic TBI patients. All patients triaged to rehabilitation facility were assessed with an index injury severe enough to warrant inpatient rehabilitation, with Rancho Los Amigos-Revised (RLA) score distributions of the following on admission to rehabilitation facility: RLA 1 (No Response, Total Assistance, N=1); RLA 2 (Generalized Response, Total Assistance, N=2), RLA 3 (Localized Response, Total Assistance, N=2), RLA 4 (Confused/Agitated, Maximal Assistance, N=1), RLA 5 (Confused, Inappropriate/Non-Agitated, Maximal Assistance, N=6), RLA 6 (Confused, Appropriate, Moderate Assistance, N=4), RLA 7 (Automatic, Appropriate, Minimal Assistance for ADLs, N=1), RLA 8 (Purposeful, Appropriate, Stand-By Assistance, N=0), RLA 9 (Purposeful, Appropriate, With Standby Assist on Request, N=0), RLA 10 (Purposeful, Appropriate, Modified Independent, N=0), RLA Unknown, (N=5). Thus, all chronic patients with known RLA were of score 7 or less, with 16 of 21 total patients (76%) needing at moderate assistance for activities of daily living (ADL) due to their brain injury (5 (24%) needing total assistance, 7 (33%) needing maximal assistance, and 4 (19%) needing moderate assistance). Hence we observe that the chronic TBI population in this study is one of overall moderate to total impairment in ADL. CT data was available for 11 of 21 chronic patients (5 extraaxial hemorrhage only, 3 intraaxial hemorrhage only, 3 both extra and intraaxial hemorrhage).

The post-injury time range from 16 to 250 days after injury, with an average of 176.4 days (or 6.4 months) post-injury (**Table 2**). Using ANOVA we show that the AutoAb[GFAP] levels were significantly elevated in chronic TBI patients (mean 15.08 ± 2.82 units, $p < 0.001$) compared to healthy controls as previously reported (mean 2.90 ± 0.93 units) (**Figure 2**).

We also plotted a graph of the plasma AutoAb[GFAP] against the time post-injury based on this set of 21 subjects. Each chronic TBI subject only had one timed plasma sample drawn as part of the TRACK-TBI Pilot study (**Figure 3**) – while the sample size is limited, no significant correlation was found between post-injury time and AutoAb[GFAP] levels (Spearman rank correlation test, data not shown).

We also examined the relationship between CT intracranial lesion and AutoAb[GFAP] levels in these chronic patients. Results on ANOVA showed no statistically significant differences across the 4 categories ((Mean ± SE): extraaxial only, 14.32 ± 6.01; intraaxial only, 8.27 ± 2.88; both extra- and intraaxial, 13.82 ± 7.98, unknown CT pathology, 13.07 ± 3.86; $p = 0.168$).

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DISCUSSION

In this study, we expand on our previous finding that there is a dominant anti-GFAP autoantibody response within 5-10 days among a subset of severe TBI subjects.[26,39] While the number of plasma samples is still relatively small within the cohort, the TRACK-TBI Pilot dataset was selected for this study because it is well-characterized with 13 published manuscripts regarding various components of these TBI subjects across the full range of TBI severity – including proteomic and genetic biomarkers, neuroimaging, and outcome data.[31,36,37,40-43] Based on the 217 subjects with available biosamples from this cohort, we identified that anti-GFAP autoantibody levels were elevated in acute plasma samples from brain injury subjects who had self-reported history of prior TBI with or without LOC when compared to acute TBI subjects without self-reported prior TBI (**Figure 1**). There is no correlation between GFAP antigen levels and GFAP autoantibody levels in these acute samples and to initial GCS. We also found no statistically significant differences between AutoAb[GFAP] and acute CT pathology – widely utilized as the current clinical standard for TBI diagnosis and a surrogate marker of brain injury after acute TBI.[1,44-46] Since newly acquired anti-GFAP antibody response usually takes about 5 days to manifest,[26,47,48] it is unlikely that the acute post-TBI autoantibody levels we reported here were due to de novo response to current TBI, but rather to a sustained increase due to prior head injuries. However, at present we cannot rule out if the acute TBI event might serve to be an antigen-boosting event for those subjects with preexisting anti-GFAP antibody titers. It is also interesting to consider that repeated mTBI/concussion can potentially serve as an autoantigen-boosting event.

Our study is the first to report AutoAb[GFAP] values across the spectrum of acute TBI. The reason for anti-GFAP reactivity in a subset of normal controls is not completely known. We have previously reported similar results in our first study on AutoAb[GFAP].[26] We also noted that autoantibodies to other human autoantigens have been reported in normal populations.[49,50] We

suspect that the baseline anti-GFAP autoantibody levels we observed in certain normal controls likely reflect the TBI health history of those subjects e.g. they may have experienced previous unreported concussions or other subclinical neurological events.[25] It is also presently unclear as to why AutoAb[GFAP] were statistically significantly elevated in those with an acute TBI and history of prior TBI when compared to acute TBI without prior history of TBI, but not normal controls. However, in the samples captured from the auto rehabilitation cohort with confirmed prior TBI did demonstrate a statistically higher GFAP autoantibody levels. Whether this contradiction is reflective of the small sample size, a high prevalence of unreported TBI in the control group, and/or combination thereof remains to be determined. It is also possible that GFAP autoantibody level represent not only initial injury severity/mortality, but also individual variability in the immune response and/or clearance of autoantibodies. Hence, our study should be considered preliminary and future studies with serial collection of GFAP autoantibodies are therefore needed to better quantitate the time course in individuals to better characterize his hypothesized variability.

All samples were collected within 24 h following the current TBI event and thus the plasma AutoAb[GFAP] we measured in these acute TBI subjects likely reflect prior brain injury or perturbation incidents. Patients reporting prior TBI without LOC had a slightly lower AutoAb[GFAP] level on average than those reporting prior TBI with LOC. This preliminarily suggests that the severity of prior exposure exerts some effect on the magnitude of the AutoAb[GFAP] response measureable in plasma. However, future studies with a larger population of post-TBI patients in which initial injury characteristics are available is needed to further validate this finding.

While preliminary, this is the first report of significant plasma AutoAb[GFAP] elevation in TBI patients at the chronic time point (mean > 6 month) compared to age-matched controls (**Figure**

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2). Since the autoantibody response is a marker of sustained immunological memory, it may be the case that neuronal or glial autoantibody biomarkers can be useful to confirm a diagnosis of chronic TBI in cases where history is vague or incomplete.

Some of the limitations of the current study are as follows. Currently, we focused on IgG responses; in future studies, we plan to examine in parallel IgM-based autoantibody responses to investigate acute changes. To increase the throughput of the anti-GFAP autoantibody assays, it will be desirable to use microplate-based enzyme-linked immunosorbent assays (ELISAs); we are working towards this direction. Additionally, owing to institutional-specific differences in medical record documentation, all prior injury information was patient-reported and additional injury characteristics (e.g. acute GCS in the rehabilitation setting) could not be independently confirmed and/or clarified. Another limitation is the lack of longitudinal blood samples within the same patient and thus we were unable to follow the temporal profile of AutoAb[GFAP] response. To this end, we will be expanding our AutoAb[GFAP] studies to the ongoing, NIH-funded prospective multicenter TRACK-TBI study[51] with acute (day 1, 3, 5), subacute (2 weeks) and chronic (6 month) blood samples from up to 2,700 TBI subjects across injury severities, as well as 300 non-TBI controls, as part of the U.S. Department of Defense (DOD) TBI Endpoints Development (TED) Initiative.[52] Data from these future studies will allow us to examine whether elevations of post-injury AutoAb[GFAP] associate with patient outcome.

Conclusion

In conclusion, AutoAb[GFAP] assays may be useful to study the dynamic interactions amongst brain autoimmune mechanisms post-TBI across acute and chronic injury settings. There are two important new findings reported in this study: (i) We find that in the setting of acute TBI, plasma AutoAb[GFAP] levels associate with a history of past exposure to TBI; (ii) Further, this is the first study to report elevated AutoAb[GFAP] levels at a chronic time point (average of 6 months post-

injury) among moderate to severe TBI patients. With emerging attention on reexamining TBI as a chronic condition with various comorbidities[3,38,53-56] we can now add brain protein-targeting autoantibodies to a growing list of potential useful biomarkers for studying at-risk acute and chronic TBI populations.

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ACKNOWLEDGMENTS

INTEREST DECLARATION

K.K.W. holds stocks of Banyan Biomarkers, Inc., a company interested in commercialization of diagnostic tests for TBI.

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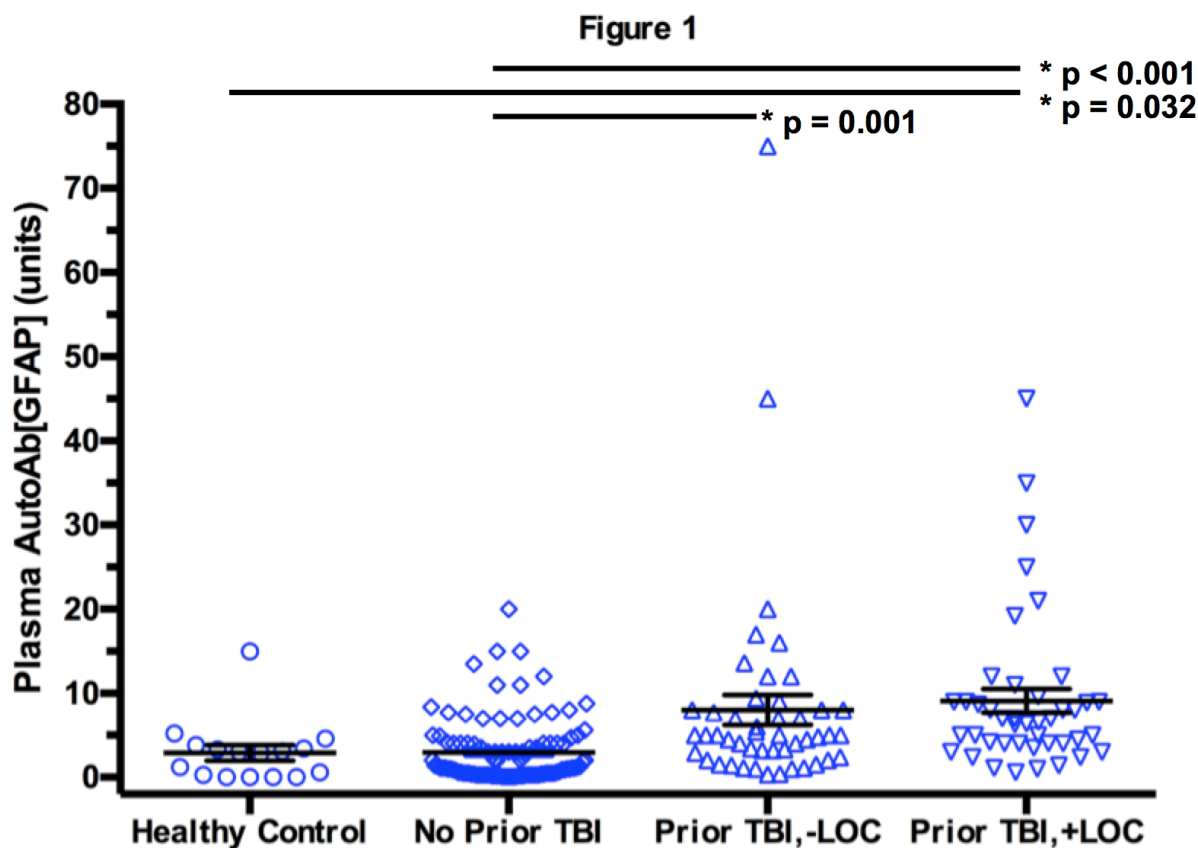
Figure 1.

Caption: Mean and SEM are shown for each respective patient subgroup (healthy control, acute TBI reporting no prior TBI, acute TBI reporting prior TBI without LOC, acute TBI reporting prior TBI with LOC). The plasma AutoAb[GFAP] is shown units as described in the Methods section of the manuscript. Statistically significant differences across subgroups are denoted with (*) and the respective p-value. AutoAb[GFAP] = glial fibrillary acidic protein auto-antibody; LOC = loss of consciousness; SEM = standard error of the mean; TBI = traumatic brain injury.
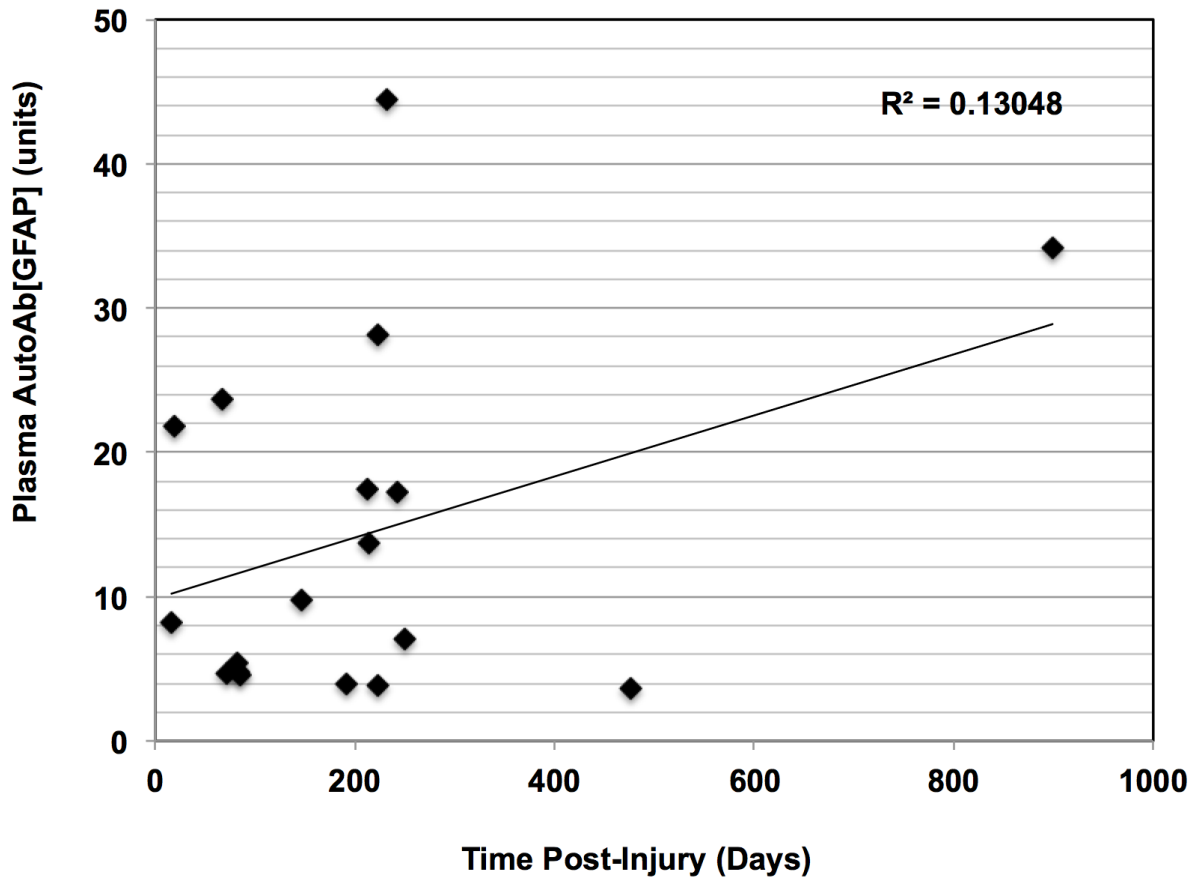
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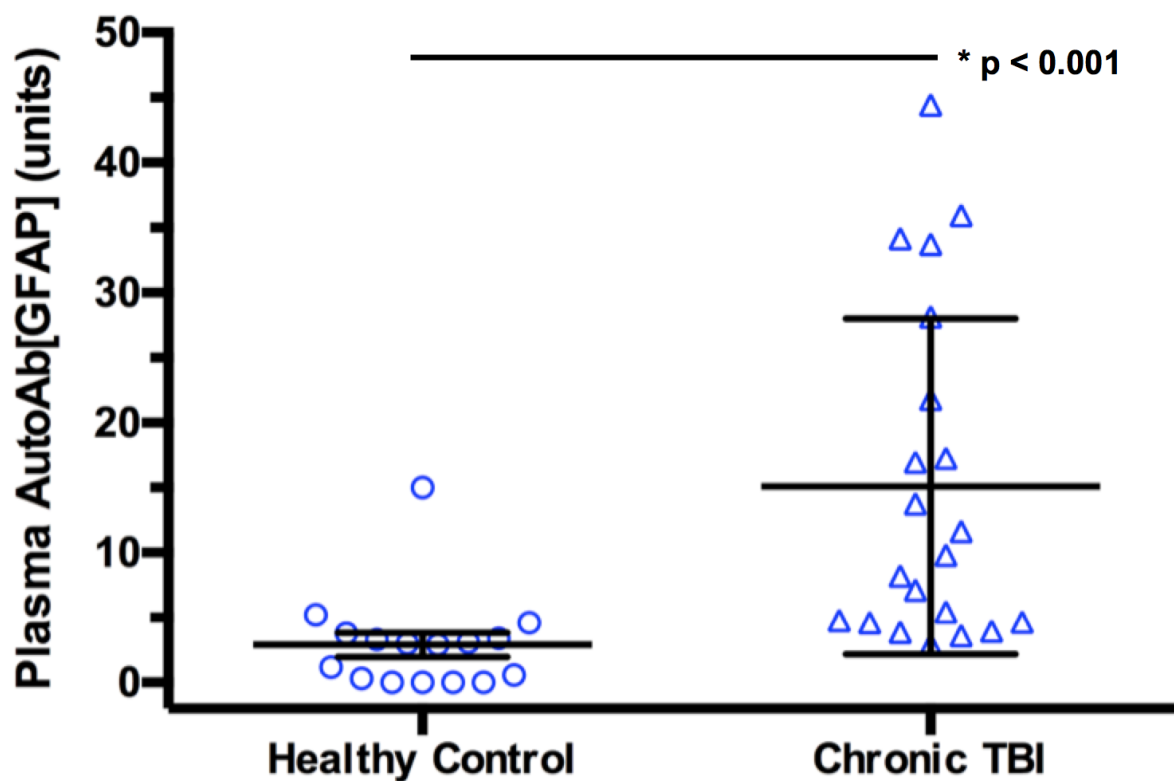
Figure 2

Figure 2.

Caption: Mean and SEM are shown for healthy control versus chronic TBI patients. The plasma AutoAb[GFAP] is shown in units as described in the Methods section of the manuscript. Statistically significant differences across subgroups are denoted with (*) and the respective p-value. AutoAb[GFAP] = glial fibrillary acidic protein auto-antibody; LOC = loss of consciousness; SEM = standard error of the mean; TBI = traumatic brain injury.

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Figure 3.

Figure 3.

Caption: Scatterplot for plasma AutoAb[GFAP] plotted against time post-injury for 21 chronic TBI patients. The plasma AutoAb[GFAP] is shown in units as described in the Methods section of the manuscript. The correlation coefficient (R^2) is shown. AutoAb[GFAP] = glial fibrillary acidic protein auto-antibody; TBI = traumatic brain injury.

Supplementary Figure 1.

Caption: Workflow of the manifold-immunoblot method to quantifying human AutoAb[GFAP] levels in plasma samples. Alk. Phos. = Alkaline Phosphatase; AutoAb[GFAP] = glial fibrillary acidic protein auto-antibody; BCIP-NBT = 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride; IgG = immunoglobulin G; MW = molecular weight; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Table 1. Demographics and Injury Characteristics of TBI Subjects

|	**Acute TBI**	**Chronic TBI**
Age	**N=196**	**N=21**
Mean, SD	42.4, 17.8	44.4, 20.5
Range	16-86	19-81
Gender	**N=196**	**N=21**
Male	151 (73%)	16 (76%)
Female	55 (27%)	5 (24%)
GCS	**N=196**	**N=21**
3-8	12 (6%)	---
9-12	6 (3%)	---
13-15	160 (82%)	---
Unknown	18 (9%)	---
Prior TBI	**N=196**	**N=21**
None	106 (54%)	4 (19%)
Yes, without LOC	47 (24%)	4 (19%)
Yes, with LOC	43 (22%)	13 (62%)
Admission Head CT	**N=196**	**N=21**
Negative	108 (55%)	---
Extraaxial Only	22 (11%)	5 (24%)
Intraaxial Only	24 (12%)	3 (14%)
Extra + Intraaxial	42 (21%)	3 (14%)
Unknown	---	10 (48%)
Outcome (6-month)	**N=137**	**N=17**
GOSE = 1	7 (5%)	0 (0%)
GOSE = 2	1 (1%)	0 (0%)
GOSE = 3	10 (7%)	1 (5%)
GOSE = 4	4 (2%)	5 (24%)
GOSE = 5	5 (10%)	5 (24%)
GOSE = 6	21 (15%)	1 (5%)
GOSE = 7	39 (28%)	1 (5%)
GOSE = 8	44 (32%)	4 (19%)

Caption: GCS data was unavailable for chronic TBI patients. CT pathology was positive for all chronic TBI patients with CT data. CT = computed tomography; GCS = Glasgow Coma Scale; GOSE = Glasgow Outcome Scale-Extended; SD = standard deviation; TBI = traumatic brain injury.

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Table 2. Plasma AutoAb[GFAP] Levels in TBI Subjects

	Acute TBI			Chronic TBI		
Post-Injury Time	N=196			N=21		
Mean ± SD	10.6 ± 6.3 (hours)			176.4 ± 44.5 (days)		
Range	0.5 to 23.9 (hours)			16.0 to 250.0 (days)		
GCS	N	Mean (SE)	Sig. (*p*)	N	Mean (SE)	Sig. (*p*)
3-8	12	3.35 (0.87)		---	---	
9-12	6	4.37 (1.59)	0.136	---	---	---
13-15	120	6.16 (0.72)		---	---	
Unknown	18	1.73 (2.35)		---	---	
Prior TBI	N	Mean (SE)	Sig. (*p*)	N	Mean (SE)	Sig. (*p*)
None	106	2.97 (0.37) [a]		4	13.25 (2.43)	
Yes, without LOC	47	8.01 (1.80) [b]	<0.001	4	15.41 (6.56)	0.956
Yes, with LOC	43	9.11 (1.42) [b]		13	15.54 (4.18)	
Admission Head CT	N	Mean (SE)	Sig. (*p*)	N	Mean (SE)	Sig. (*p*)
Negative	108	6.48 (0.87)		---	---	
Extraaxial Only	22	5.02 (2.01)		5	14.32 (6.01)	
Intraaxial Only	24	5.72 (2.04)	0.197	3	8.27 (2.88)	0.168
Extra + Intraaxial	42	3.22 (0.49)		3	13.82 (7.98)	
Unknown	---	---		10	13.07 (3.86)	

Caption: Blood draw for GFAP-AutoAb post-injury time calculated from time of injury. GCS data was unavailable for chronic TBI patients. CT pathology was positive for all chronic TBI patients with CT data. [a] and [b] denote statistically significant subgroups on Tukey's post-hoc test. CT = computed tomography; GCS = Glasgow Coma Scale; GFAP-AutoAb = glial fibrillary acidic protein auto-antibody; SD = standard deviation; TBI = traumatic brain injury. SE, standard error of the mean.

Supplementary Figure 1.
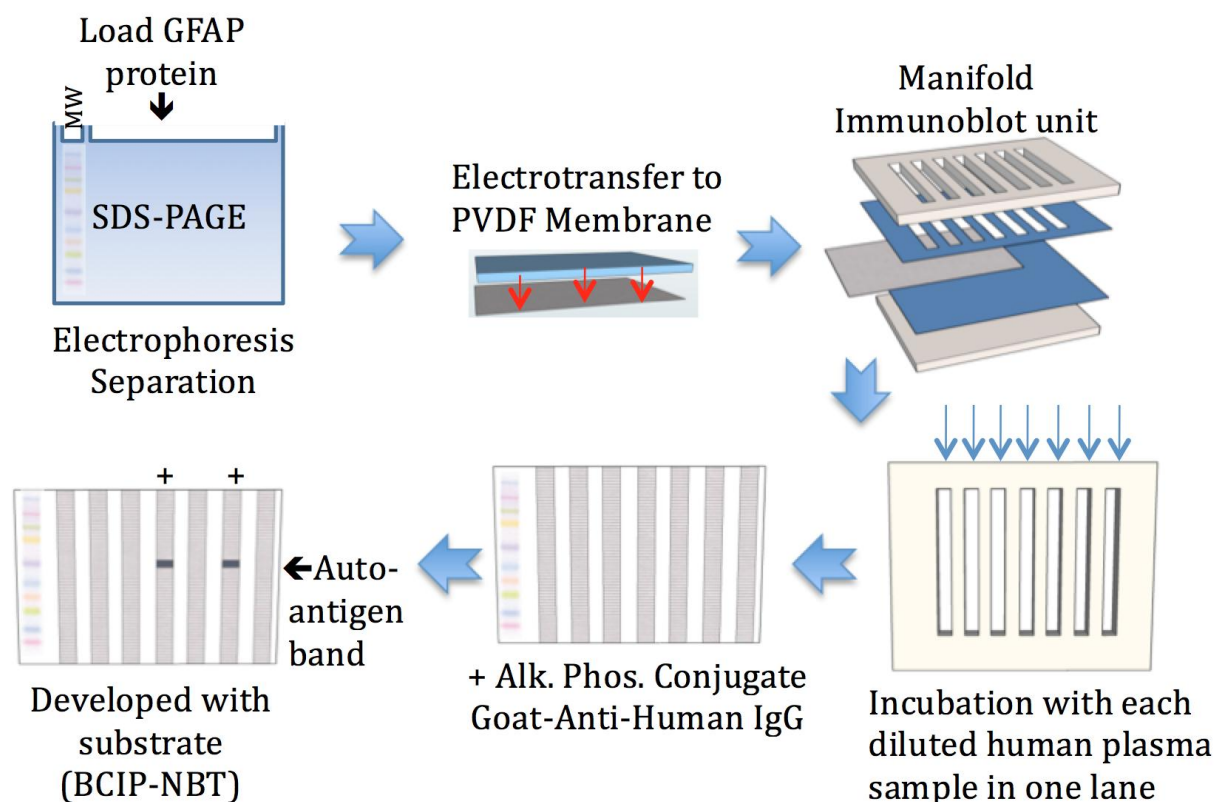
Workflow of the manifold-immunoblot method to quantifying human AutoAb[GFAP] levels in plasma samples. Alk. Phos. = Alkaline Phosphatase; AutoAb[GFAP] = glial fibrillary acidic protein auto-antibody; BCIP-NBT = 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride; IgG = immunoglobulin G; MW = molecular weight; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

ORIGINAL ARTICLE

COMT Val^{158}Met polymorphism is associated with nonverbal cognition following mild traumatic brain injury

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Kevin K. W. Wang[12] · Pratik Mukherjee[2,9] · Alex B. Valadka[13] · David O. Okonkwo[11] ·
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Abstract Mild traumatic brain injury (mTBI) results in variable clinical outcomes, which may be influenced by genetic variation. A single-nucleotide polymorphism in catechol-o-methyltransferase (COMT), an enzyme which degrades catecholamine neurotransmitters, may influence cognitive deficits following moderate and/or severe head trauma. However, this has been disputed, and its role in mTBI has not been studied. Here, we utilize the Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot (TRACK-TBI Pilot) study to investigate whether the COMT Val^{158}Met polymorphism influences outcome on a cognitive battery 6 months following mTBI—Wechsler Adult Intelligence Test Processing Speed Index Composite Score (WAIS-PSI), Trail Making Test (TMT) Trail B minus Trail A time, and California Verbal Learning Test, Second Edition Trial 1–5 Standard Score (CVLT-II). All patients had an emergency department Glasgow Coma Scale (GCS) of 13–15, no acute intracranial pathology on head CT, and no polytrauma as defined by an Abbreviated Injury Scale (AIS) score of ≥3 in any extracranial region. Results in 100 subjects aged 40.9 (SD 15.2) years

The TRACK-TBI Investigators are listed in the Appendix in alphabetical order by last name

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($COMT$ Met^{158}/Met^{158} 29 %, Met^{158}/Val^{158} 47 %, Val^{158}/Val^{158} 24 %) show that the $COMT$ Met^{158} allele (mean 101.6±SE 2.1) associates with higher nonverbal processing speed on the WAIS-PSI when compared to Val^{158}/Val^{158} homozygotes (93.8±SE 3.0) after controlling for demographics and injury severity (mean increase 7.9 points, 95 % CI [1.4 to 14.3], $p=0.017$). The $COMT$ $Val^{158}Met$ polymorphism did not associate with mental flexibility on the TMT or with verbal learning on the CVLT-II. Hence, $COMT$ $Val^{158}Met$ may preferentially modulate nonverbal cognition following uncomplicated mTBI.

Registry: ClinicalTrials.gov Identifier NCT01565551

Introduction

Traumatic brain injury (TBI)—defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force—is a comparatively common insult with variable outcomes [1, 2]. In the USA alone, at least 2.5 million people suffer TBIs annually [3], and it has been estimated that up to 5.3 million people are currently living with TBI-related disability [4]. TBI is frequently subdivided on the basis of injury severity into severe, moderate, and mild injury categories as defined by a Glasgow Coma Scale (GCS) score of 8 or less, 9-to-12, or 13-to-15, respectively [5, 6]. Although more severe injuries may disproportionately contribute to disability, the vast majority—70 to 90 %—of all TBI is characterized as "mild TBI" (mTBI) [7]. Within mTBI, considerable variability in outcome exists across individuals. Most make a complete recovery following mTBI [8, 9]; however, up to 20 % of patients experience persistent symptoms and/or cognitive or neuropsychiatric deficits [10]. Individuals with nearly identical injuries often manifest different symptoms, follow different clinical trajectories, and/or have varied functional outcomes [11]. Efforts are therefore needed to better identify those at greatest risk for posttraumatic sequela to better prognosticate and facilitate development of tailored therapy [1].

Studies have begun to investigate relationships between genetic variants within a number of candidate genes and outcome following TBI in an effort to elucidate such variability. One form of this variance—called single nucleotide polymorphisms (SNPs)—is comprised of single nucleotide substitutions arising within a gene's coding sequence and/or regulatory elements which may influence either protein structure/function or abundance, respectively. Numerous polymorphisms have been identified [12–14], but those arising within genes encoding important proteins underlying neurotransmission are thought to play an influential role in the preservation and/or impairment in cognition following TBI [15]. Catechol-

O-methyltransferase (COMT; encoded by the gene $COMT$ on chromosome 22q11.2) represents one such molecule [16–18] and is an enzyme which inactivates catecholamine neurotransmitters, e.g., dopamine (DA), epinephrine, and norepinephrine, through 3-O-methylation of the benzene ring [19]. In brain regions important to cognition, e.g., the prefrontal cortex (PFC), low expression of DA reuptake transporters makes COMT inactivation the predominant regulator of dopaminergic synaptic transmission [19–21].

A relatively common SNP arising within the coding sequence at codon 158—known as $COMT$ $Val^{158}Met$ (rs4680)—results in substitution of a methionine for valine at this position [19]. This substitution lessens the activity of COMT resulting in higher levels of dopamine in the PFC [22], and it has been shown that Val^{158}/Val^{158} individuals are up to four times more efficient at catabolizing catecholamines than Met^{158}/Met^{158} homozygotes [23]. In turn, higher bioavailability of catecholamines in the PFC in Met^{158}/Met^{158} subjects has been shown to confer a cognitive advantage over Val^{158}-carriers [24], and the Met^{158} allele is generally associated with an advantage in measures of memory, executive function, and tasks requiring attention [18, 25].

Cognitive symptoms, including memory loss, inattention, and impulsivity, are relatively common in TBI and are among the most debilitating consequences of TBI and may influence functional outcome [26]. A number of prior studies have suggested that disruption and/or dysregulation of dopaminergic transmission in the PFC may contribute to the pathogenesis of posttraumatic cognitive impairment [27]. Conversely, it has been suggested in other studies that the dopaminergic system may be pharmacologically targeted to ameliorate persistent cognitive deficits following TBI [28]. Despite its importance in modulating PFC neurotransmission, studies examining the relationship between the $COMT$ $Val^{158}Met$ polymorphism and cognitive deficits following TBI have largely been equivocal [16–18]. To date, these studies have been limited to more severe injury, and whether the $COMT$ $Val^{158}Met$ polymorphism influences posttraumatic cognitive deficits following mTBI has yet to be studied.

Here, we utilize the Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot (TRACK-TBI Pilot) dataset, a database of demographic history, biomarkers, neuroimaging, and neuropsychiatric and neurocognitive outcomes obtained at three clinical sites [29], to evaluate whether the $COMT$ $Val^{158}Met$ polymorphism influences cognitive performance 6 months following mTBI on a battery of three standardized tests—Wechsler Adult Intelligence Scale Fourth Edition Processing Speed Index subscale, Trail Making Test, and the California Verbal Learning Test Second Edition. We hypothesized that the $COMT$ $Val^{158}Met$ polymorphism is associated with improved cognitive performance following mTBI. Our data demonstrates that the $COMT$ $Val^{158}Met$ polymorphism associates with cognitive performance in select

domains, e.g., nonverbal processing speed, but not others, e.g., mental flexibility or verbal learning.

Materials and methods

Study design

The TRACK-TBI Pilot Study is a multicenter prospective observational study conducted at three Level 1 trauma centers in USA—San Francisco General Hospital, University of Pittsburgh Medical Center, and University Medical Center Brackenridge (UMCB) in Austin, Texas [29]—using the National Institutes of Health (NIH) and National Institute of Neurological Disorders and Stroke (NINDS) common data elements (CDEs) [30–33]. Inclusion criteria for the pilot study were adult patients presenting to a Level 1 trauma center with external force trauma to the head and clinically indicated head computed tomography (CT) scan within 24 h of injury. Exclusion criteria were pregnancy, comorbid life-threatening disease, incarceration, suicidal ideation/on psychiatric hold, and non-English speakers due to limitations in participation with outcome assessments. For the present study, our goal was to study the associations between $COMT\ Val^{158}Met$ and cognition after isolated and uncomplicated mTBI. Therefore, our analysis was restricted to a subset of patients with a GCS ≥ 13, no skull fracture, or acute intracranial pathology—defined as the absence of intraparenchymal contusions or hemorrhage, intraventricular hemorrhage, epidural hematoma, acute subdural hematoma, or traumatic subarachnoid hemorrhage—on non-contrasted head CT within 24 h of injury, no polytrauma as defined by an Abbreviated Injury Scale (AIS) score ≥ 3 in any extracranial body region [34, 35], as well as no prior history of cerebrovascular accident or transient ischemic attack, brain tumor, schizophrenia, learning disability or developmental delay.

Eligible subjects were enrolled through convenience sampling at all three sites. Institutional review board approval was obtained at all participating sites. Informed consent was obtained for all subjects prior to enrollment in the study. For patients unable to provide consent due to their injury, consent was obtained from their legally authorized representative (LAR). Patients were then reconsented if cognitively able at later inpatient and/or outpatient follow-up assessments for continued participation in the study.

Biospecimen acquisition and genotyping

Specimen acquisition was performed as previously described [29]. In brief, blood samples for DNA genotyping analysis were collected via peripheral venipuncture or existing peripheral venous indwelling catheters within 24 h of injury. Samples were collected in BD Vacutainer K_2-EDTA vacutainer tubes, and subsequently aliquoted and frozen in cryotubes at $-80\ °C$ within 1 h of collection in accordance with recommendations from the NIH-CDE Biomarkers Working Group [Manley 2010]. DNA was extracted from isolated leukocytes using the Wizard® Genomic DNA Purification Kit as described by the manufacturer (Promega, Madison, WI) and reported in our previous work [36]. $COMT\ Val158Met$ polymorphism ($rs4680$) was genotyped utilizing the TaqMan®SNP Genotyping Assay as described by the manufacturer (Applied Biosystems, Carlsbad, CA, Assay ID# C_25746809_50). For the purpose of evaluating a potential protective benefit of the Met^{158} allele, Met^{158}/Met^{158} and Met^{158}/Val^{158} were combined as a single group as previously described for $COMT$ [37–40] and other genetic polymorphisms in TBI [41–43]. Therefore, for data reporting and all figures, this group is referred to as Met^{158}.

Neuropsychiatric testing and outcome parameters

The NINDS defines measures of neuropsychological impairment as those "of neuropsychological functions, such as attention, memory, and executive function which are very sensitive to effects of TBI that affect everyday activities and social role participation [33]." To evaluate for neuropsychological impairment, all participants underwent outcome assessments at 6 months following TBI with a battery of NIH NINDS-designated "Core Measures"—those deemed most relevant and applicable across large TBI studies. For the current analysis, all three measures of the "Neuropsychological Impairment" domain of the outcome CDEs were included:

Wechsler Adult Intelligence Scale, fourth edition Processing Speed Index Subscale

The Wechsler Adult Intelligence Scale, fourth edition Processing Speed Index Subscale (WAIS-PSI) is a summary measure of nonverbal processing speed and is comprised of two nonverbal tasks (symbol search and coding) which require visual attention and motor speed [44]. In studies of TBI, it has been shown to predominately reflect impairment in perceptual processing speed with a small component attributable to working memory and only minimal contribution from motor speed [45]. The composite score is scalar, ranging from 50 to 150 to correspond to the 0.1st to 99.9th percentile of performance across age groups. Scores of ~90, 100, and ~110 correspond to the 25th, 50th, and 75th percentiles, respectively [44].

Trail Making Test

The Trail Making Test (TMT) is a two-part timed test (TMT-A and TMT-B), and both scores are measured in number of seconds needed for the patient to complete the task. TMT-A assesses visual processing, and TMT-B assesses mental

flexibility and processing speed [46]. In order to derive a purer index of executive control and mental flexibility separate from visual processing and motor speed, we used the difference score between the Trial B and Trial A (TMT B-A) as previously described [47–49]. In this test, a lower score suggests improved performance.

California Verbal Learning Test, second edition

The California Verbal Learning Test, second edition (CVLT-II) is a verbal learning and memory task in which five learning trials, an interference trial, an immediate recall trial, and a post-20 min recall trial are performed. The CVLT-II trials 1–5 Standard Score is a summative score of the first five learning trials normed for age and sex and provides a global index of verbal learning ability [50]. The CVLT-II was substituted for the Rey Auditory Verbal Learning Test (RAVLT) listed in the NIH NINDS outcome CDEs due to relevant revisions of the second edition and higher consistency on between-norm sets [51].

Statistical analysis

Group differences in patient demographics and mechanism of injury across $COMT$ Met^{158} carriers versus Val^{158}/Val^{158} homozygotes were assessed by Pearson's chi-squared test (X^2) for categorical variables and analysis of variance (ANOVA) for continuous variables. Fisher's exact test was used to assess for differences in categorical variables with group counts ≤5. Means and standard deviations are reported for continuous descriptive variables. Group differences are reported between $COMT$ genotype and each outcome measure using ANOVA. Multivariable linear regression was performed for each of the three outcome measures to adjust for age and education years as recommended [44–46, 49, 50]; the WAIS-PSI Composite Score and CVLT-II trials 1–5 Standard Score are already age-normed and thus further adjusted only for education years, while the TMT B-A score was further adjusted for age and education years. As this is a study of mTBI, the GCS was used to adjust for injury severity (GCS 15 vs. less than 15). The adjusted unstandardized coefficient of regression (B) and associated standard error (SE) was used to quantify mean increase or decrease in the outcome measure associated with a per-unit increase in a continuous predictor or a change in the subcategory of a categorical predictor. All multivariable regression models conformed to tests for goodness-of-fit. To account for race stratification, race was entered onto the multivariable regression with three subcategories to include the two largest race categories (Caucasian, African-American/African) as well as a third category of aggregated "other races" for races with small (<5) group counts. Significance was assessed at $\alpha=0.05$. All analyses were performed using Statistical Package for the Social Sciences (SPSS) v.22 (IBM Corporation, Chicago, IL). Figures were constructed with GraphPad Prism v.6 (GraphPad Software, La Jolla, CA).

Results

Patient demographics and mechanisms of injury

In total, the present study included 100 subjects (Table 1). Overall, subjects had a mean age of 40.9 years (SD 15.2) and were 66 % male. The race distribution was 70 % Caucasian, 14 % African American/African, 5 % Asian, 1 % American Indian/Alaskan Native, 1 % Hawaiian/Pacific Islander, and 9 % more than one race. Subjects had a mean of 14.2 years of education (SD 2.9). Mechanisms of injury were 33 % fall, 26 % motor vehicle crash, 22 % pedestrian versus auto, 15 % assault, and 4 % struck by/against object. GCS distribution was 3, 20, and 77 % for GCS of 13, 14, and 15, respectively. Distribution of admission GCS did not change with respect to genotype. For injury severity classification, GCS of 13 and 14 were combined into a single group of "GCS less than 15". There was also no difference in posttraumatic amnesia—another important predictor for posttraumatic cognitive impairment—across genotypes [11, 52–54]. In total, 66 subjects were discharged from the emergency department (ED), 30 were admitted to the hospital ward, and 4 were admitted to the intensive care unit (ICU). No statistically significant difference in ED disposition was observed across genotypes (Table 1).

$COMT$ genotype distribution was 29 % Met^{158}/Met^{158} ($n=29$), 47 % Met^{158}/Val^{158} ($n=47$), and 24 % Val^{158}/Val^{158} ($n=24$). $COMT$ allelic frequencies (A=0.53, G=0.47) were not found to deviate significantly from Hardy-Weinberg equilibrium ($X^2=0.33$, $p=0.566$). Years of education were higher for Met^{158} carriers than for Val^{158}/Val^{158} homozygotes ($p=0.016$), and a higher prevalence of Val^{158}/Val^{158} homozygotes was noted in African-American/African subjects ($p=0.042$). No other significant differences were observed in the distribution of each demographic and clinical descriptor across $COMT$ Met^{158} and Val^{158}/Val^{158} genotypes (Table 1).

Outcome measures

We first assessed whether the $COMT$ $Val^{158}Met$ polymorphism was associated with divergent performance on three primary cognitive measures—WAIS-PSI, TMT B-A, and CVLT-II—following isolated, uncomplicated mTBI. $COMT$ Met^{158} carriers showed significantly higher nonverbal processing speed on WAIS-PSI when compared to $COMT$ Val^{158}/Val^{158} homozygotes (Met^{158} 103.8±13.3; Val^{158}/Val^{158} 94.1±15.7; $p=0.004$) (Table 2). $COMT$ Met^{158} subjects did not associate with a task requiring mental flexibility on TMT B-A (Met^{158} 46.6±51.5; Val^{158}/Val^{158} 63.8±42.0, $p=$

Table 1 Demographic and clinical information of included subjects with mild traumatic brain injury

Variable	COMT Met[158] (N=76)	COMT Val[158]/Val[158] (N=24)	Sig. (p)
Age (years)			
Mean±SD	40.5±15.7	42.2±14.1	0.643
Gender			
Male	49 (65 %)	17 (71 %)	0.566
Female	27 (35 %)	7 (29 %)	
Race			
Caucasian	57 (81 %) [a]	13 (19 %) [a]	0.042
African-American/African	7 (50 %) [a]	7 (50 %) [b]	
Other races	12 (75 %) [a]	4 (25 %) [a]	
Education (years)			
Mean±SD	14.6±2.7	13.0±3.1	0.015
Mechanism of injury			
Motor vehicle crash	24 (32 %)	2 (8 %)	0.110
Pedestrian versus auto	17 (22 %)	5 (21 %)	
Fall	23 (30 %)	10 (42 %)	
Assault	9 (12 %)	6 (25 %)	
Struck by/against object	3 (4 %)	1 (4 %)	
Posttraumatic amnesia			
No	30 (40 %)	11 (46 %)	
Yes	42 (55 %)	10 (42 %)	0.310
Unknown	4 (5 %)	3 12 %)	
GCS—field[a]			
<15	21 (36 %)	6 (35 %)	0.982
=15	38 (64 %)	11 (65 %)	
GCS—ED arrival			
<15	19 (25 %)	4 (17 %)	0.579
=15	57 (75 %)	20 (83 %)	
ED disposition			
ED discharge	53 (70 %)	13 (54 %)	0.284
Hospital ward admission	20 (26 %)	10 (42 %)	
ICU admission	3 (4 %)	1 (4 %)	

Race distributions are reported as row percentages. All other distributions reported as column percentages. The race subgroup "other races" was combined due to individual small sample sizes of Asian (N=5; Met[158] =4, Val[158]/Val[158] =1), American Indian/Alaskan Native (N=1; Met[158] =1), Hawaiian/Pacific Islander (N=1; Met[158] =1), and more than one race (N=9; Met[158] =6, Val[158]/Val[158] =3)

COMT catechol-O-methyltransferase, ED emergency department, GCS Glasgow Coma Scale, ICU intensive care unit, SD standard deviation

[a] Data for GCS—Field was only available for 76 patients

Table 2 Distribution of performance on 6-month cognitive outcome measures following mild traumatic brain injury by COMT genotype

Outcome Measure	Met[158] (N=76)	Val[158]/Val[158] (N=24)	Sig. (p)
WAIS-PSI Composite Score[a]	103.8±13.3	94.1±15.7	0.004
TMT Trail B minus A Time[b]	46.6±51.5	63.8±42.0	0.139
CVLT-II Trial 1–5 Standard Score[a]	54.5±11.1	53.7±9.4	0.740

Distributions are reported as mean±standard deviation

COMT catechol-O-methyltransferase, CVLT-II California Verbal Learning Test, second edition, TMT Trail Making Test, WAIS-PSI Wechsler Adult Intelligence Scale, fourth edition, Processing Speed Index

[a] Higher scores suggest improved performance

[b] Lower scores suggest improved performance

0.139) (Table 2). *COMT Val*[158]*Met* polymorphism did not associate with verbal learning and fluency as measured by the CVLT-II Trial 1–5 Standard Score (*Met*[158] 54.5±11.1; *Val*[158]/*Val*[158] 53.7±9.4, $p=0.740$) (Table 2).

COMT Val[158]*Met* is associated with nonverbal processing speed after mTBI

To further assess the association between *COMT Val*[158]*Met* and nonverbal processing speed as measured by the WAIS-PSI composite score, multivariable regression was performed to control for education years, race, and injury severity (Table 3). *COMT Met*[158] carriers demonstrated higher adjusted mean scores on WAIS-PSI (101.6±2.1) compared to their *Val*[158]/*Val*[158] counterparts (93.8±3.0), which corresponds to a mean increase of 7.9 points (95 % CI [1.4 to 14.3], $p=0.017$) (Fig. 1). Consistent with prior reports [55–57], education years associated with WAIS-PSI ($B=1.4$, 95 % CI [0.4 to 2.3], $p=0.005$). Greater injury severity also associated with a decrease in nonverbal processing speed (GCS 15, 101.6±1.9; GCS <15, 93.8±3.0; $B=-7.9$, 95 % CI [−14.1 to −1.7], $p=0.013$). Race did not show a significant association with WAIS-PSI ($p=0.539$) on multivariable analysis. Further, multivariable subgroup analysis performed in the Caucasian group—the largest group—demonstrated a statistical trend between the *COMT Val*[158]*Met* polymorphism and performance on WAIS-PSI ($B=7.5$, 95 % CI [−1.1 to 16.0], $p=0.086$). Future studies are needed to confirm this finding in a larger population.

COMT Val[158]*Met* is not associated with mental flexibility after mTBI

To further assess the association between *COMT Val*[158]*Met* and mental flexibility as measured by the TMT B-A time, multivariable regression was performed to control for education years, race, and injury severity. Since the TMT B-A has not been intrinsically adjusted for age, we further adjusted for age in the current analysis. *COMT Val*[158]*Met* did not demonstrate an association with TMT B-A after adjustment (*Met*[158] 47.7±7.1; *Val*[158]/*Val*[158] 58.8±10.2; $B=-11.1$, 95 % CI [−33.0 to 10.8], $p=0.318$) (Table 3). Consistent with prior reports [58, 59], both age years ($B=1.2$, 95 % CI [0.6 to 1.8], $p<0.001$) and education years ($B=-5.2$, 95 % CI [−8.4 to −2.0], $p=0.002$) associated with decreased and increased performance on mental flexibility, respectively. Injury severity did not show a significant association with TMT B-A (GCS 15 47.5±6.5; GCS <15 59.0±10.3; $B=11.5$, 95 % CI [−9.7 to 32.6], $p=0.284$). Race did not show a significant association with TMT B-A ($p=0.492$) on multivariable analysis.

COMT Met[158] is not associated with verbal learning after mTBI

To further assess the association between *COMT Val*[158]*Met* and verbal learning as measured by the CVLT-II, multivariable regression was performed to control for education years, race, and injury severity. *COMT Val*[158]*Met* did not demonstrate an association with CVLT-II after adjustment (*Met*[158] 50.9±1.6; *Val*[158]/*Val*[158] 51.6±2.4; $B=-0.7$, 95 % CI [−5.8 to 4.3], $p=0.771$) (Table 3). Consistent with prior reports [60], education years ($B=0.6$, 95 % CI [−0.1 to 1.4], $p=0.098$) showed a borderline association with verbal learning. Greater injury severity also associated with a decrease in verbal learning (GCS 15 53.7±1.5; GCS <15 48.7±2.4; $B=-5.0$, 95 % CI [−9.9 to −0.1], $p=0.044$). Race showed a borderline significant association with CVLT-II ($p=0.068$) on multivariable analysis, driven primarily by a difference between the Caucasian subgroup and the heterogeneous "other races" subgroup ($B=-5.9$ [−11.5 to −0.2], $p=0.042$).

Discussion

In the present study, we sought to investigate whether the *COMT Val*[158]*Met* polymorphism is associated with cognitive performance at 6 months following mild closed head injury in an isolated, uncomplicated mTBI population. We found that subjects with the *COMT Met*[158] allele showed higher performance on a measure of nonverbal processing speed compared to *Val*[158]/*Val*[158] homozygotes at 6 months following injury independent of injury severity and race. We also demonstrate that the *COMT Val*[158]*Met* polymorphism is not associated with a measure of executive control and mental flexibility or a measure of verbal learning after controlling for injury severity and race. We confirm that greater injury severity is associated with poorer nonverbal processing speed and verbal learning. Further, racial stratification was not found to significantly associate with nonverbal processing speed, mental flexibility, or verbal learning after uncomplicated mTBI in the current patient population.

In our current analysis, *COMT Met*[158] carriers showed an adjusted mean score of 101.6 on the WAIS-PSI, while *Val*[158]/*Val*[158] homozygotes showed 93.8—these scores correspond to the ~55th percentile and the ~34th percentile of nonverbal processing speed performance in the normal population, respectively [44]. We also find that the adjusted mean scores (~50 s) on the CVLT-II correspond to the general mean of the normal population for both *COMT Val*[158]*Met* groups [50]. Further, the adjusted TMT B-A times for both *COMT* groups fall within the means reported in literature (~40 to ~60) for the normal/uninjured population [49, 61, 62]. Thus, it is worth noting that a subgroup of patients with isolated uncomplicated mTBI demonstrates heightened risk

Table 3 Multivariable analysis of the *COMT Val^{158}Met* polymorphism and 6-month cognitive outcome following mild traumatic brain injury

WAIS-PSI Composite Score[a]	Mean±SE	B [95 % CI]		Sig. (*p*)
COMT Val^{158}Met				0.017
Val^{158}/Val^{158}	93.8±3.0	Reference		–
Met^{158}	101.6±2.1	7.9 [1.4, 14.3]		
GCS				0.013
GCS=15	101.6±1.9	Reference		–
GCS <15	93.8±3.0	−7.9 [−14.1, −1.7]		
Race				0.539
Caucasian	96.8±2.1	Reference		–
African-American/African	95.8±3.6	−1.1 [−9.0, 6.9]		0.790
Other	100.5±3.5	3.7 [−3.5, 10.9]		0.312
Education (years)	–	1.4 [0.4, 2.3]		0.005
TMT Trail B minus A Time[b]	Mean±SE	B [95 % CI]		Sig. (*p*)
COMT Val^{158}Met				0.318
Val^{158}/Val^{158}	58.8±10.2	Reference		–
Met^{158}	47.7±7.1	−11.1 [−33.0, 10.8]		
GCS				0.284
GCS=15	47.5±6.5	Reference		–
GCS <15	59.0±10.3	11.5 [−9.7, 32.6]		
Race				0.492
Caucasian	59.2±7.1	Reference		–
African-American/African	43.0±12.3	−16.2 [−43.1, 10.7]		0.235
Other	57.4±12.2	−1.8 [−27.0, 23.4]		0.888
Education (years)	–	−5.2 [−8.4, −2.0]		0.002
Age (years)	–	1.2 [0.6, 1.8]		<0.001
CVLT-II Trial 1–5 Standard Score[a]	Mean±SE	B [95 % CI]		Sig. (*p*)
COMT Val^{158}Met				0.771
Val^{158}/Val^{158}	51.6±2.4	Reference		–
Met^{158}	50.9±1.6	−0.7 [−5.8, 4.3]		
GCS				0.044
GCS =15	53.7±1.5	Reference		–
GCS <15	48.7±2.4	−5.0 [−9.9, −0.1]		
Race				0.068
Caucasian	54.7±1.6	Reference		–
African-American	50.1±2.8	−4.7 [−10.9, 1.5]		0.139
Other	48.9±2.8	−5.9 [−11.5, −0.2]		0.042
Education (years)	–	0.6 [−0.1, 1.4]		0.098

The WAIS Processing Speed Index (WAIS-PSI) Composite Score and the CVLT-II Trial 1–5 Standard Score are adjusted for education years, race (Caucasian, African-American/African, other races), and GCS (15 vs. less than 15). The TMT Trail B minus A Time is adjusted for age, education years, race, and GCS. Distributions are reported as adjusted mean±standard error. The mean difference (*B*) between *COMT Met^{158}* and *COMT Val^{158}/Val^{158}* and associated 95 % CI is reported for each outcome measure CVLT-II, California Verbal Learning Test, Second Edition; TMT, Trail Making Test; WAIS, Wechsler Adult Intelligence Scale, Fourth Edition.

CI confidence interval, *COMT* catechol-O-methyltransferase, *CVLT-II* California Verbal Learning Test, second edition, *GCS* Glasgow Coma Scale, *TMT* Trail Making Test, *WAIS* Wechsler Adult Intelligence Test

[a] Higher scores suggest improved performance

[b] Lower scores suggest improved performance

for decreased performance on nonverbal processing, but not verbal learning or executive function at 6 months postinjury, and this subgroup associates with the common SNP *COMT Val^{158}Met*.
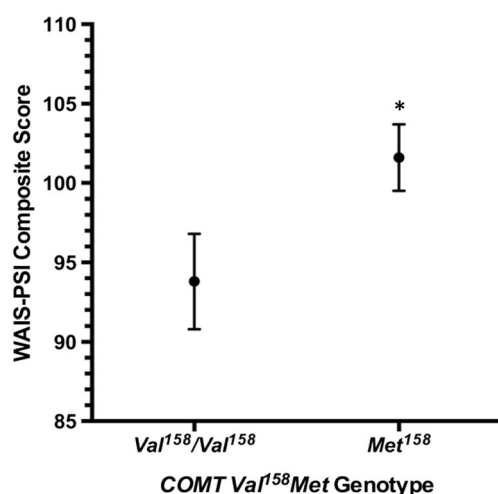
Fig. 1 *COMT Val^158^Met* and 6-month WAIS-PSI Composite Score after mild traumatic brain injury. The *COMT Val^158^Met* polymorphism is associated with statistically greater preservation of nonverbal processing speed 6 months following mild traumatic brain injury after adjusting for race, years of education, and injury severity. Means and standard errors on the WAIS-PSI Composite Score are shown for *Met^158^* and *Val158/Val^158^* genotype groups. *COMT*, Catechol-O-Methyltransferase, *WAIS-PSI* Wechsler Adult Intelligence Scale Fourth Edition—Processing Speed Index. $*p<0.05$.

It is generally accepted that acute physiologic recovery occurs by 6 months post-mTBI on imaging studies [9, 63, 64], and studies report that most cognitive symptoms resolve by within the first 3 months in mTBI [65, 66]. To our knowledge, this is the first study of the association between *COMT Val^158^Met* and cognitive performance at an extended time point of recovery, such as 6 months following mTBI. Prior reports examining the potential influence of the *COMT Val^158^-Met* polymorphism on TBI cognitive outcomes have been conducted during acute and subacute recovery with a mean time of collection within 2 months postinjury and have been predominately limited to patients with moderate and/or severe injuries [17, 18, 67]. For example, in a cohort of 113 TBI rehabilitation patients assessed at a mean of 2 months postinjury,[17] *Val^158^/Val^158^* homozygotes were found to score lower on a measure of cognitive flexibility—the ability to alter a behavioral response against changing contingencies [68]—and to have a greater number of perseverative errors. In another sample of 32 moderate-to-severe TBI patients with 40 health controls, *COMT Met^158^* was found to associate with preserved strategic control of attention at 2 months postinjury [67]. In the largest study of *COMT* and moderate-to-severe TBI to date, Willmott et al. did not find an association between *COMT* and measures of cognition at roughly 1 month postinjury [18]. However, this study evaluated cognitive performance at a time point that was not standardized and closer to the time of injury (mean 29 days); the authors suggest that cognitive assessment at 6–12 months postinjury may be more likely to detect subtle group differences as demonstrated in the present report.

There is physiological evidence in support of a potential modulatory role of the *COMT Met^158^* allele in cognitive performance following TBI. The PFC is a key center for overall executive function, attention, and strategic planning [69–71], in which its rich dopaminergic pathways are more dependent on COMT for regulation and modulation at the synaptic cleft [19–21]. Prior studies have demonstrated that the *COMT Val^158^Met* polymorphism is associated with differences in cognitive performance in the absence of brain injury [23, 72]. Given the absence of measures of baseline preinjury performance in our population or neuropsychiatric data in appropriately uninjured age-matched controls, we cannot conclude whether our results reflect the maintenance of preexisting cognitive differences between genotypes and/or an altered trajectory of recovery or impairment following mTBI.

There are also several additional limitations to the present study. Our data was obtained for a relatively small sample size ($n=100$) in a predominately Caucasian male population and did not conform to known HapMap Phase III subpopulations; therefore, there is a need for studies of confirmation in similar populations and of validation in larger and more diverse study populations. We also included patients only with isolated mTBI in the absence of intracranial findings on CT and a limited period of diminished consciousness and/or posttraumatic amnesia; thus, the generalizability of our results is limited. We also include no neuroimaging outside of 24 h or magnetic resonance imaging. Therefore, it is possible that a subset of the subjects developed delayed pathology on neuroimaging and would no longer be classified as uncomplicated. We pursued analyses designed to investigate a hypothesized relationship between the *COMT Val^158^Met* polymorphism and cognitive outcome and did not explore the structure-function implications of *COMT* with specific brain pathology or variables important to the trajectory of recovery such as treatment and support. There is also a need to examine gene-gene interaction with other susceptibility loci in the context of mTBI to better elucidate complex interactions and mechanisms through which the *COMT* molecular pathway may influence response and recovery to TBI. Finally, all of our findings must be considered preliminary until they are formally replicated.

Conclusions

The *COMT Val^158^Met* polymorphism (*rs4680*) is associated with nonverbal cognitive performance following uncomplicated mTBI without polytrauma. More specifically, the *COMT Met^158^* allele is associated with increased performance in nonverbal processing speed, while no associations were seen on mental flexibility or verbal learning. Larger studies in similar populations will be of value to confirm the role of *COMT Val^158^Met* polymorphism in these domains and to explore its effects in other cognitive domains following mTBI.

Whether *COMT Val^158/Val^158* homozygotes would benefit from heightened clinical surveillance and/or pharmacologic and cognitive behavior therapy remains to be determined and may represent an important direction of future studies.

Compliance with ethical standards

Conflicts of interest   The authors declare that they have no conflicts of interest.

Research involving human participants   All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent   Informed consent was obtained from all individual participants included in the study.

Appendix

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